THE UNIVERSITY OF ALBERTA

DIAZONIUM-1-H-TETRAZOLE MODIFICATION OF PHOSPHORYLASE

by

(C) WARREN CONRAD BREIDENBACH III

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

DEPARTMENT OF BIOCHEMISTRY

EDMONTON, ALBERTA

FALL, 1972

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ABSTRACT

Diazonium-1-H-tetrazole (DHT) was used to examine reactive residues in phosphorylases. Treatment of both phosphorylase a and b with this reagent resulted in inactivation. The conditions employed for treatment of the protein determined the per cent inactivation and specificity of the reaction. At room temperature the concentration of DHT necessary to inactivate phosphorylase b was greater than that required at 0°C. Varying the pH at 0°C changed the amount of DHT required to inactivate the enzyme and specificity of the reaction. More DHT was required to inactivate the enzyme at pH 8.8 than at pH 6.1. This was due to the rapid hydrolysis of DHT and modification of residues not essential for catalytic activity.

Titration of the phosphorylases at 0°C and at both pH 6.1 and 8.8 results in a drop in activity during the first minute, but no substantial decrease during the remaining 30 minutes, even though sufficient DHT is present to continue the inactivation. Radioactive titration with phosphorylase b indicated that this phenomenon was due to titration of residues essential for catalytic activity during the first minute, followed by titration of residues not essential for catalytic activity during the remaining 30 minutes. If the concentration of reactants was lowered then the rate of inactivation could be slowed sufficiently so that residues essential for catalytic activity took longer than one minute to react completely.

Therefore, by controlling temperature, pH, concentration of reactants, and the length of time that the reaction proceeds, conditions were found where only residues essential for catalytic activity were modified. Treatment of phosphorylase b, 5 - 10 mg/ml, with 0.3 to 0.5 mM DHT, at pH 6.1, 0°C for one minute, results in modification of 0.8 to 1.2 residues per enzyme monomer with 70 - 100% loss in activity. Absorption spectra of these samples showed a peak at 320 nm and a shoulder at 387 nm with molar absorptivity values of about 1.0×10^4 and 2.0×10^3, respectively.

Model compound studies indicated that this reagent reacts with tyrosine, histidine, lysine, tryptophan, cysteine, arginine, serine and threonine residues. Comparison of the model compound absorption spectra with the spectral properties of the DHT-treated phosphorylase b indicated that the modified residue was not likely to be a tyrosine, histidine, lysine or tryptophan. Studies with IAM-treated DHT phosphorylase b implied that none of the reactive sulfhydryl residues were titrated. Emission spectra of PLP isolated from DHT-treated phosphorylase b showed that the cofactor was not modified.

Due to technical difficulties molar absorptivity values were not obtained for arginine, serine or threonine. Therefore, nothing definite can be said about the possibility that one of these may be the reactive residue.

Protection studies with substrates did not give conclusive evidence as to whether or not the DHT modified a

residue at the active site.

In conclusion, DHT under specified conditions modifies one residue per phosphorylase b monomer producing 100% inactivation. The nature of this residue is still unknown.

ACKNOWLEDGEMENTS

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I would like to thank Mr. Morris Aarbo for operation of the analytical ultracentrifuge, and Mr. Michael Nattriss for operation of the amino acid analyzer. Thanks are also extended to Miss Katharine Hurlburt for her assistance during one summer.

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I would also like to thank Dr. Howard Engers for planning and assisting in the experiments reported in Part III, Section B, and Part IV, Section C, Number 2.

My thanks are also extended to Dr. Margaret-Ann Armour who carefully read the manuscript, correcting my many abuses of the English language. I would also like to thank Miss Jaclyn Dorsey for her capable typing.

Finally, the excellent supervision of Dr. N.B. Madsen is acknowledged with gratitude.

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LIST OF ABBREVIATIONS

DHT - Diazonium-1-H-tetrazole

5AT - 5-Aminotetrazole

GP - β-Glycerophosphate

ME - 2-Mercaptoethanol

EDTA - Ethylenediamine Tetraacetic Acid

IAM - Iodoacetamide

P_i - Potassium phosphate

NAT - N-Acetyltyrosine

DTT - Dithiothreitol

c.p.m. - counts per minute

d.p.m. - disintegrations per minute

PLP - Pyridoxal-5'-Phosphate

PMP - Pyridoxamine-5'-Phosphate

SDS - Sodium Dodecyl Sulfate

AMP - Adenosine 5'-Phosphate

ε - Molar absorptivity

IAM phosphorylase - refers to iodoacetamide treated phosphorylase such that the B_1 and B_2 sulfhydryl residues were reacted.

DHT phosphorylase - refers to phosphorylase treated with DHT. Note that the abbreviation DHT was often used to represent the diazonium-1-H-tetrazole derivative incorporated into the protein.

Note: When referring to the protein the order of the abbreviations in each case was determined by the order in which

the enzyme was treated. For example, apo DHT IAM phosphorylase b refers to a phosphorylase b sample first treated with iodoacetamide, then with diazonium-1-H-tetrazole, and finally resolved.

INTRODUCTION

The first synthesis of 5-aminotetrazole was carried out by Thiele (1). This was followed by diazotization of 5-aminotetrazole forming diazonium-1-H-tetrazole (2). It has been known for many years that diazonium salts could react with amino acid residues (3, 4, 5); however, it was not until seventy years after its original synthesis that DHT was used by Horinishi et al. to modify histidine and tyrosine residues in proteins (6). Vallee et al. improved on the method of Horinishi. He showed that for quantitative results all modified histidine and tyrosine residues should be bisazo derivatives, since the spectra of monoazotyrosine and monoazohistidine overlap (7).

A number of workers started to use this reagent, following the procedure outlined by Vallee et al., to examine tyrosine and histidine content in different proteins. Horinishi et al. repeated their previous work with insulin, this time taking into consideration the overlapping spectra of modified residues (8). Further work on insulin using DHT was done by Shibata et al. (9). Muramatu et al. examined the effect of DHT on trypsin. They were able to modify tyrosyl, histidyl and lysyl residues and relate their importance to catalytic activity (10). Zaheer et al. used DHT to examine tyrosyl and histidyl content and examine their relation to catalytic activity in RNA polymerase from E. coli (11).

DHT was used by some workers as an active site directed reagent. Bak et al. modified glucose dehydrogenase from

A. oryzae and concluded that one histidyl residue probably occurs at the glucose binding site (12). Shimada et al. using DHT were able to show that one tyrosine was essential to myosin A - adenosine triphosphatase activity (13). DHT was used by Pfleiderer et al. to show that histidyl residues were at the active site of aminopeptidase M (14). Ohtsuki et al. modified with DHT one histidyl residue of subtilisin BPN'. This residue was essential for catalytic activity and thought to be in the active site of subtilisin BPN' (15). Vallee et al. used DHT to show that carboxypeptidase A contained one histidyl residue essential for peptidase, but not esterase activity (16).

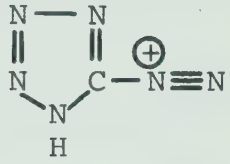
In some of the above mentioned work, it was overlooked that DHT was not a group specific reagent. DHT has been reported to be capable of modifying tyrosine, histidine, lysine, cysteine, arginine, serine and threonine residues in proteins (13, 16). Tyrosine and histidine modification could be determined easily by spectral changes. Acid hydrolysis destroys DHT modified tyrosine, histidine, lysine, and to a small extent arginine (7 , 16). Hence amino acid analysis would indicate which of these residues were modified. However, no method exists for the determination of serine, threonine or cysteine residues which were modified. Furthermore, this thesis will show that DHT may also modify tryptophan residues in proteins, and that no method for its determination exists.

The products which DHT is expected to form with each of

the amino acid residues in a protein are shown below:

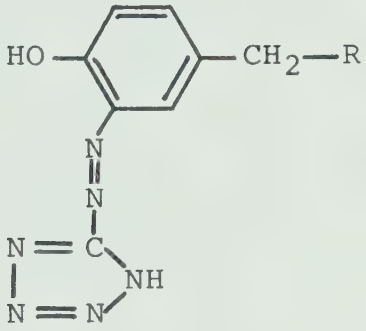
SCHEME I

Diazonium-1-H-tetrazole

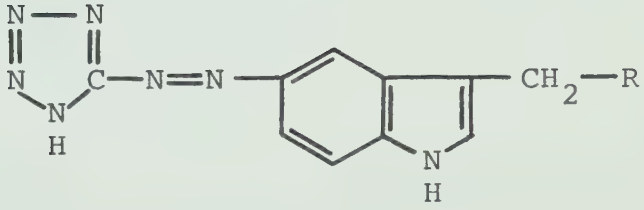


+ amino acid residue yields with:

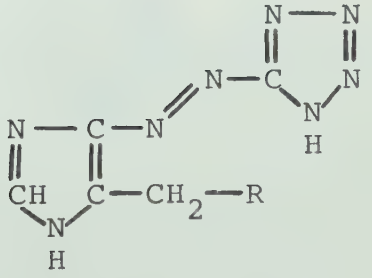
Tyrosine:



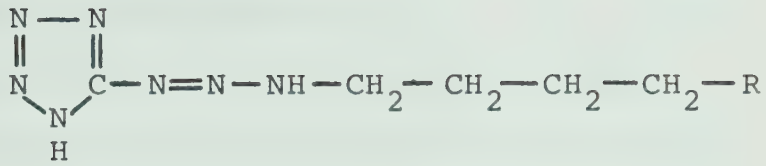
Tryptophan:



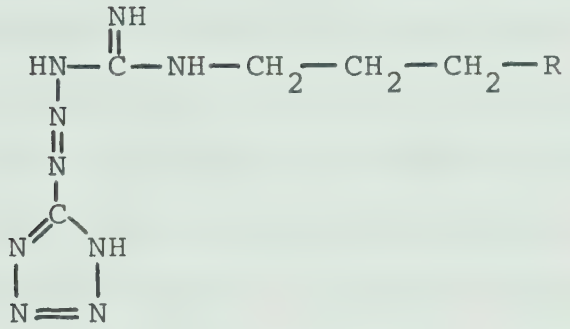
Histidine:



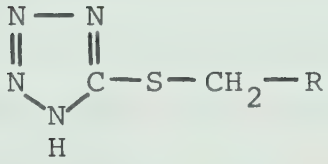
Lysine:



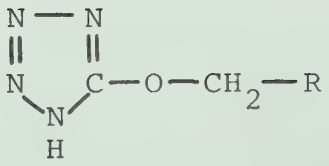
Arginine:



Cysteine:



Serine or Threonine:



where R is the polypeptide backbone of the protein.

It should also be noted that tyrosine, tryptophan and histidine may also form bisazo products.

Except for the work with sulfhydryl groups, little has been done in the way of examining reactive residues in phosphorylase a or b. Huang et al. used cyanate to examine lysine residues in both proteins, finding that modification of 52 residues per molecule of a and 23 per molecule of b resulted in total loss of catalytic activity (17). Philip et al. also examined lysine residues in phosphorylase with fluorodinitrobenzene (18). Fukui et al. used glyoxal to modify lysine and arginine residues in phosphorylase b (19), while Wang et al. used glutaraldehyde to modify lysine residues (20). No evidence was found to suggest that any of these amino acids were directly involved in the catalytic process. For further discussion of the extensive literature on glycogen phosphorylase, the reader is referred to the comprehensive review by Fischer et al. (21).

MATERIALS AND METHODS

Crystalline rabbit muscle phosphorylase b was prepared as described by Fischer and Krebs (22) and crystalline phosphorylase a was prepared by the method of Green and Cori (23) except that mercaptoethanol or dithiothreitol replaced cysteine in both procedures. The enzymes were recrystallized at least three times. Immediately before use, phosphorylase b or a was passed through a Sephadex G-25 gel filtration column pre-equilibrated with the appropriate buffer.

Rabbit liver glycogen, purchased from Sigma Chemical Co., was purified by passage through a Dowex 1-Cl column. Sodium dodecyl sulfate, AMP, N-acetyl-L-arginine, and N-acetyl-L-histidine were also purchased from Sigma Chemical Co. Glucose-1-phosphate was purchased from Raylo. 5-Aminotetrazole monohydrate was purchased from Aldrich Chemical Co., while ^{14}C-5-aminotetrazole monohydrate was purchased from Schwarz. This radioactive 5AT (500 μC, 0.023 mmoles) was dissolved in 1 ml of 1.6 M HCl and stored below 0°C. ^{14}C-n-Hexadecane was purchased from Amersham. Crystalline sodium nitrite was purchased from Fisher. The N-acetyl derivatives of L-tyrosine, L-serine, L-cysteine, L-lysine and L-tryptophan were purchased from Calbiochem.

Phosphorylase activity was measured in the direction of glycogen synthesis according to Cori et al. (24), using 20 mM GP, 20 mM ME, 1.5 mM EDTA, pH 6.8, as the buffer to dilute the enzyme to the appropriate protein concentration. The substrate in the reaction was 16 mM glucose-1-phosphate,

1% glycogen and 1 mM AMP. Reactions with phosphorylase *a* were run in the presence and absence of AMP.

Protein concentration was determined from the absorbance at 280 nm, using a Zeiss PMQIII spectrophotometer and a value for $E_{1\%}$ of 13.2 (25). For DHT treated proteins, the method of Lowry *et al.* (26) was employed.

Reduced phosphorylase was prepared by the method of Fischer *et al.* (27). Preparation of apophosphorylase *b* and its reconstitution were carried out according to the procedure of Fischer *et al.* (28). PLP content in phosphorylase *b* was determined according to the procedure of Shaltiel *et al.* (29).

IAM titration of the B_1 and B_2 sulfhydryl groups in phosphorylase *b* was carried out according to the procedure of Hasinoff *et al.* (30). When the protein was inactivated by treatment with IAM, i.e., when the B_1, B_2, N and A sulfhydryl groups were titrated, the procedure of Battell *et al.* was followed (31).

DHT was prepared in the following manner. 5-Amino-tetrazole (0.100 g) was dissolved in 2.3 ml of 1.6 M HCl. Sodium nitrite (0.070 g) was dissolved in 1.7 ml of distilled H_2O. Both solutions were put into an ice bath at 0°C. After equilibration, the $NaNO_2$ solution was added to the 5AT solution with stirring. The reaction was allowed to proceed with stirring for 8 minutes, after which time distilled H_2O at 0°C was added to dilute the DHT to the required concentration. Potassium hydroxide (4 M) was added to adjust the pH to a value of about 4.5. The DHT was maintained at 0°C

with stirring until its use.

Radioactive DHT was prepared in a similar manner. Non-radioactive 5AT (0.100 g) was dissolved in 2.3 ml of 1.6 M HCl. This solution (0.23 ml) was added to 0.1 ml of the radioactive 5AT (2.3 μmole). This served as the radioactive 5AT stock solution used to prepare DHT. Sodium nitrite (0.070 g) was dissolved in 1 ml of distilled H_2O. The temperatures of this solution and the radioactive 5AT were lowered to 0°C in an ice bath. The $NaNO_2$ solution (0.12 ml) was added to the radioactive 5AT with stirring. The reaction was allowed to proceed with stirring for 8 minutes, after which time distilled H_2O at 0°C was added to dilute the DHT to the appropriate concentration. Potassium hydroxide (4 M) was added to adjust the pH to a value of about 4.5. The DHT was maintained at 0°C with stirring until its use. It should be noted that concentrated solutions of DHT are highly explosive. The reagent should be handled with extreme care and only in dilute solutions. Solutions above 0.2 M should not be used.

DHT was assayed with N-acetyltyrosine. N-acetyltyrosine (0.2232 g) was dissolved in 10 ml of 0.67 M $KHCO_3$, pH 8.8. This 0.1 M N-acetyltyrosine solution was stored below 0°C. When it was desired to assay DHT, an appropriate aliquot from the stock solution of N-acetyltyrosine was diluted in 0.67 M $KHCO_3$, pH 8.8, and to this the DHT was added. Appropriate concentrations were employed so that N-acetyltyrosine was always at least 100X in excess of DHT. The reaction was allowed to

proceed for 30 minutes at which time the absorbance at 478 nm was measured. Using a molar absorptivity value of $\varepsilon = 5.1 \times 10^3$, the amount of monoazotetrazole-N-acetyltyrosine formed, and hence the amount of DHT present initially, was calculated.

Phosphorylase a and b samples in 50 mM potassium phosphate, 1.5 mM EDTA, pH 6.1, or 80 mM borate, 1.5 mM EDTA, pH 8.8, were treated with DHT. The protein was dialyzed or passed through a G-25 Sephadex column into the former buffer, while it was always passed through a G-25 Sephadex column into the latter buffer. When dialyzed into 50 mM P_i, 1.5 mM EDTA, pH 6.1, a buffer-protein ratio of at least 50:1 was employed with three buffer changes over a 12 to 20 hour period at room temperature. The enzyme was never allowed to remain in this buffer for more than 30 hours. When a column was used, the protein was passed into either borate or phosphate buffer and treated with DHT. The enzyme was never allowed to remain in borate buffer for more than 12 hours.

Once in the required buffer, an aliquot from the stock solution of DHT at 0°C was added to the protein at room temperature or 0°C. The reaction was terminated usually by the addition of a 0.5 M GP, 0.5 M ME, pH 7.0, buffer to the reaction mixture, so that the final concentration of GP and ME would be at least 10X that of the DHT. After termination of the reaction, the reaction mixture was dialyzed or passed through a G-25 Sephadex column usually into 20 mM GP, 5 mM ME, 1.5 mM EDTA, pH 6.8, in order to separate the DHT treated protein from the excess DHT. When dialyzed a buffer-protein

ratio of at least 50:1 was used with three changes over three days at 0°C.

N-acetylamino acids were treated with DHT in the following manner. The N-acetylamino acid was weighed out and added to 50 mM P_i, 1.5 mM EDTA, pH 6.1 so that the final concentration was 0.15 M. Addition of the N-acetylamino acid lowered the pH of the buffer, hence 10 N NaOH was used to return it to a value of 6.1. The N-acetylamino acid (0.15 M) in 50 mM P_i, 1.5 mM EDTA, pH 6.1, at 0°C was treated with about 1.2 mM DHT, thus assuring 100X excess of amino acid to DHT.

Ultra violet absorption spectra were obtained on a Durrum PGS spectrophotometer. Emission spectra were obtained on a Turner Model 210 fluorescence spectrophotometer.

High-voltage electrophoresis was carried out on Whatman #1 paper using 0.6 mg to 1 mg per cm. The buffers used were as follows:

pH 6.5: 879 ml deionized water, 100 ml pyridine, 3 ml glacial acetic acid. Coolant: 92% toluene, 8% pyridine by volume.

pH 1.8: 2% formic acid, 8% acetic acid. Coolant: varsol.

Side strips of electropherograms were analyzed for radioactivity using a Nuclear-Chicago Actigraph III strip scanner with 4 π geometry. Analysis was done using a slow scan speed (15 cm/hr) and long time constant (50 seconds).

The Beckman Spinco Model E analytical ultracentrifuge

was used for determination of sedimentation coefficients. A speed of 60,000 r.p.m. was used at a temperature of 20°C. The centerpiece was Kel-F. The percentage of components with different sedimentation coefficients was determined by estimation of areas of empirically resolved components.

Protein hydrolysis was carried out at 110°C in redistilled 6 N HCl. Samples of phosphorylase were dialyzed against deionized water before hydrolysis. After hydrolysis samples were analyzed on a Beckman Spinco Model 121 amino acid analyzer. The number of moles of each amino acid was determined in comparison with the value for alanine obtained by Appleman et al. (32).

Radioactive samples were counted in Aquasol or Bray's D scintillation solutions. Aquasol was purchased from Beckman. The Bray's D solution was prepared in the following manner: PPO, 5 g/liter; naphthalene, 100 g/liter; dioxane, balance of liter.

Unless otherwise stated, 0.4 to 0.7 mg of ^{14}C-DHT treated protein was added to each scintillation vial. Samples were counted on a Beckman Model 200 scintillation counter for 10 minutes and two cycles. Radioactive incorporation was calculated by counting ^{14}C-DHT treated protein after dialysis. Specific activity was determined from samples taken directly from the reaction mixture before dialysis.

Pepsin and trypsin treatment of phosphorylase b was carried out according to the procedure of Zarkadas (33).

EXPERIMENTAL RESULTS

Part I. Development of Methodology

A. DHT Assay System

In previous studies DHT concentration was determined by one of three methods: first, 100% conversion of 5AT to DHT was assumed (9); second, by adding DHT to an excess of free histidine and measuring the absorbance peak of the reaction product (6 , 13); or third, by the addition of DHT to a large excess of either N-acetyltyrosine, N-acetyltyrosine ethyl ester or N-carbobenzoxytyrosine, and measuring the absorbance peak of the reaction product (7, 8, 10, 15).

The first method is unsatisfactory. Vallee *et al*. (7) pointed out that the second method, assaying with free histidine, was inaccurate since the histidine derivative exhibits maximal absorbance between 360 nm and 380 nm, where DHT absorbance is quite intense. Therefore, Vallee developed another assay method whereby DHT was added to a 27 to 45 times molar excess of N-acetyltyrosine or N-acetyltyrosineamide, in 1 M $KHCO_3$, pH 8.8. The mixture remained at room temperature for 20-30 minutes. Under these conditions Vallee *et al*. (7) reported that the DHT reacts stoichiometrically to yield the monoazotetrazole tyrosine derivative, with an absorbance peak at 478 nm and a molar absorptivity value of 5.1×10^3. Vallee reported a 75% - 95% conversion of 5AT to DHT as determined by this assay procedure. Subsequent workers with this reagent normally used an assay procedure which was the same as, or similar to, that developed

by Vallee.

In Vallee's assay procedure there is the inherent assumption that all the DHT will react with the tyrosine derivative before it decomposes. Although he reported that "DHT decomposes--albeit slowly--and, hence, the concentration of the reagent may change on standing", he did not examine the rate of this decomposition under any conditions. Therefore, an experiment was carried out in order to determine this rate under assay conditions, i.e., 1 M $KHCO_3$, pH 8.8, at room temperature.

If DHT does decompose at a significant rate, then this pathway will compete with the desired reaction of DHT with N-acetyltyrosine. If such a competitive reaction exists,then both the length of time that DHT remains at room temperature in 1 M $KHCO_3$, pH 8.8, before addition of N-acetyltyrosine, and the amount of N-acetyltyrosine added, will affect the yield of monoazotetrazole-N-acetyltyrosine. Therefore, aliquots from a stock solution of DHT were added to a series of cuvettes each containing a different concentration of N-acetyltyrosine in 1 M $KHCO_3$, pH 8.8. The mixtures remained at room temperature for 30 minutes, at which time the absorbance at 478 nm was recorded. The results, shown in Figure 1, demonstrate that the N-acetyltyrosine:DHT ratio does affect the amount of product formed, thus indicating the presence of a competitive reaction. The experiment was repeated, but this time the DHT was added to 1 M $KHCO_3$ at pH 8.8 containing no N-acetyltyrosine. After one minute an aliquot of

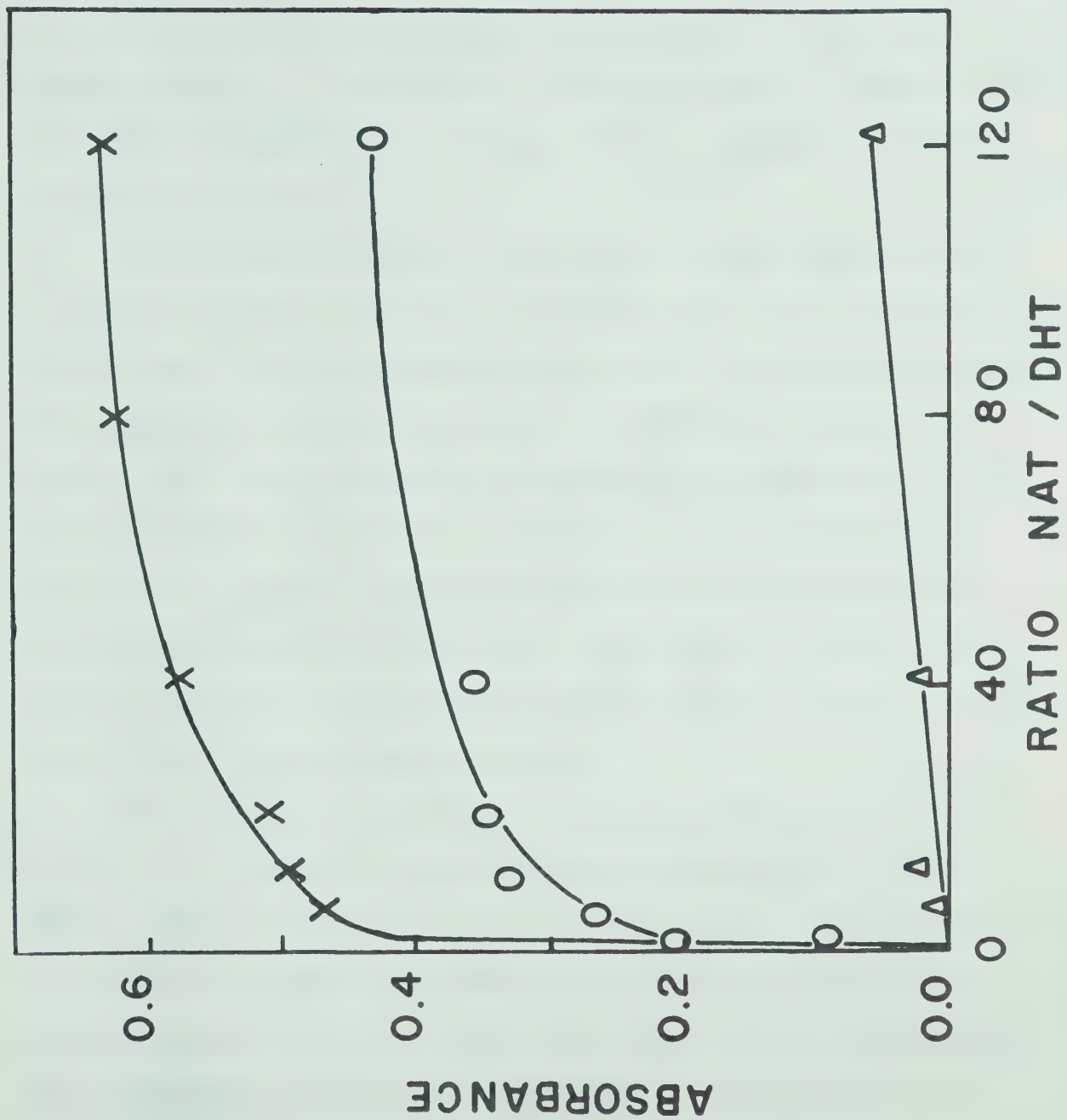
Figure 1

Effect of varying both the ratio and the time of addition of N-acetyltyrosine to DHT on formation of monoazotetrazole-N-acetyltyrosine. Plot of absorbance measured at 478 nm vs. ratio of N-acetyltyrosine to DHT. Value in parentheses represents the percentage of DHT hydrolyzed. Percentages obtained by comparing absorbance values at largest N-acetyltyrosine/DHT ratio, assuming that addition of DHT at 0 time results in 100% conversion of N-acetyltyrosine to the product.

X-X DHT added immediately (0 time) to a cuvette containing N-acetyltyrosine.

O-O Addition of DHT to cuvette followed one minute later by addition of N-acetyltyrosine (32%).

Δ-Δ Addition of DHT to cuvette followed ten minutes later by addition of N-acetyltyrosine (91%).


ABSORBANCE
0.0
0.2
0.4
0.6
0
40
80
120
RATIO NAT / DHT

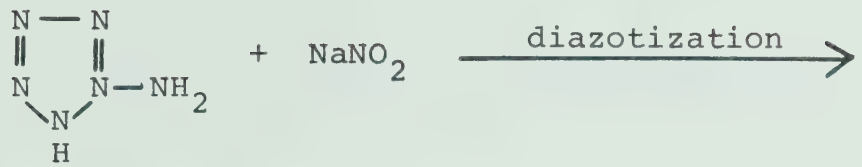
N-acetyltyrosine was added. Thirty minutes after the addition of N-acetyltyrosine the absorbance at 478 nm was recorded. The procedure was repeated, this time allowing a ten minute interval between the addition of DHT and N-acetyltyrosine. The results, shown in Figure 1, demonstrate that DHT decomposition is rapid, 32% in one minute and 90% within 10 minutes.

These results suggest that there is some competitive reaction for DHT. Since it is known that diazonium compounds hydrolyze, it may be assumed that this is the other reaction. The sequence shown in Scheme II is therefore suggested. Hence, any assay procedure employing the conditions outlined above must add a sufficient excess of N-acetyltyrosine to ensure complete conversion of DHT to monoazotetrazole-N-acetyltyrosine. As indicated in Figure 1, for stoichiometric conversion one should have at least a 100-fold excess of N-acetyltyrosine to DHT.

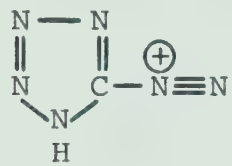
The absorbance at 478 nm resulting from the addition of DHT to a 100-fold excess of N-acetyltyrosine in 0.67 M $KHCO_3$, pH 8.8, was followed over 105 minutes. The results are shown in Figure 2. There is a large increasing absorbance within the first minute and then the absorbance slowly drifts upward. The latter phenomenon may or may not be directly related to monoazotetrazole-N-acetyltyrosine formation.

With these results in mind, the assay procedure presented in Materials and Methods was developed. DHT concentration

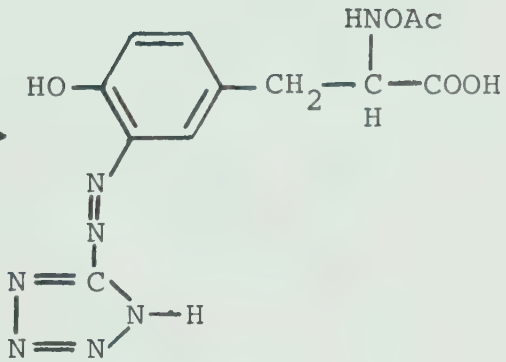
SCHEME II



(5-aminotetrazole)



(diazonium-1-H-tetrazole)

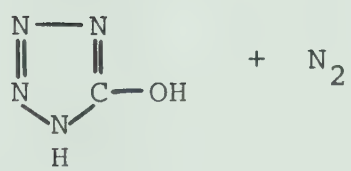


(monoazotetrazole-N-acetyl-tyrosine)

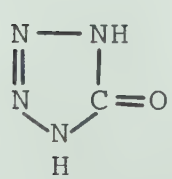
+

H_2O

(assumed reaction)



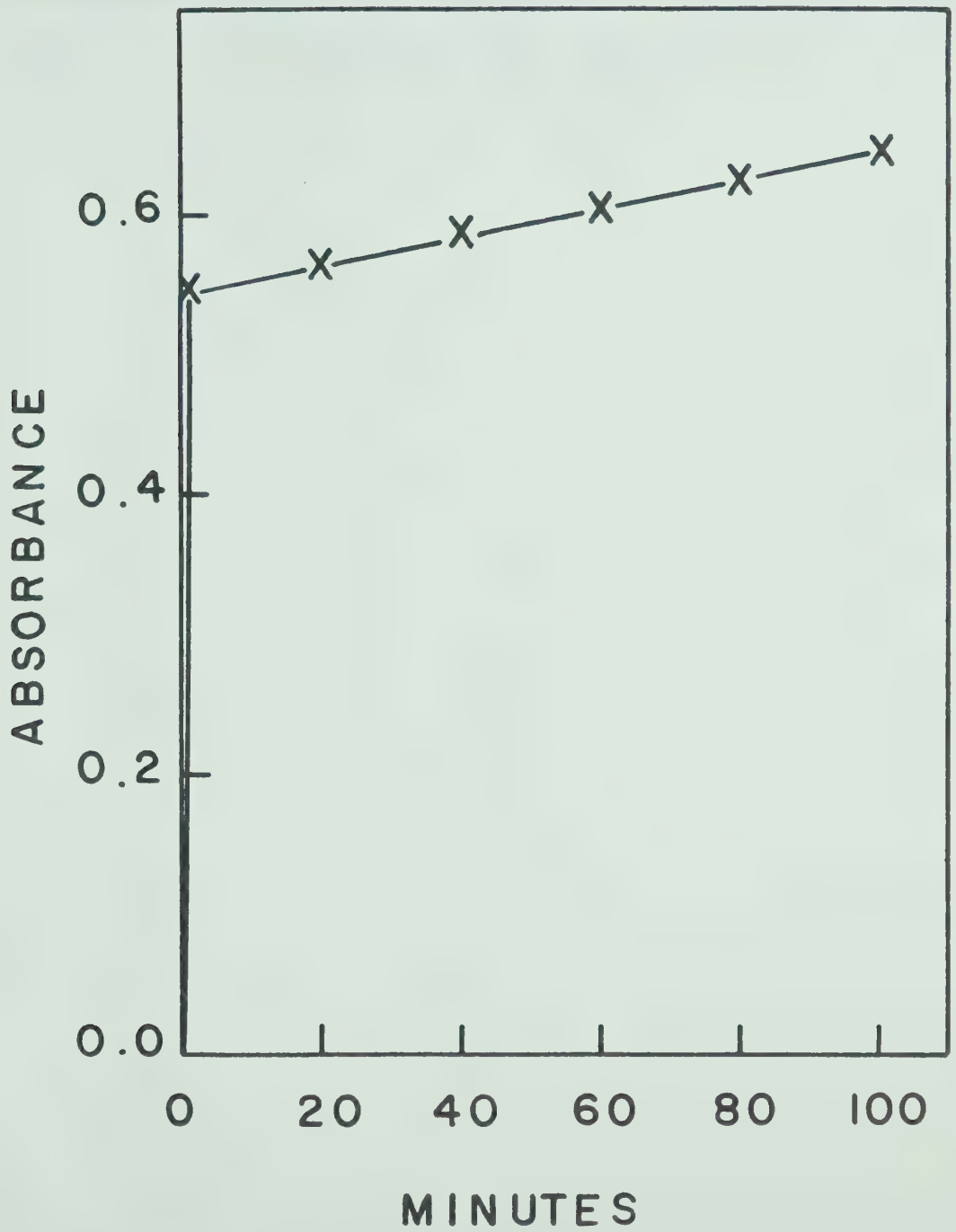
(tetrazolinone)



(5-hydroxy-1-H-tetrazole)

Figure 2

Absorbance of monoazotetrazole-N-acetyltyrosine. Absorbance of 0.116 mM monoazotetrazole-N-acetyltyrosine in 0.67 M $KHCO_3$, pH 8.8, measured at 478 nm plotted *vs.* time. Zero time was taken as the point of addition of DHT to the assay mixture.


ABSORBANCE
0.6
0.4
0.2
0.0
0
20
40
60
80
100
MINUTES

determined by this method showed 100 to 105% yield based on the initial 5AT concentration. This method therefore represents a substantial improvement over previous methods where a 75-95% yield of DHT was reported (7, 8).

B. Buffer Systems

Since the rate of reaction between DHT and amino acid residues in a protein was expected to show a pH dependence, phosphorylase samples were treated with DHT within a pH range of 6.1 to 8.8. Previous workers used $KHCO_3$ buffer at pH 8.8, since it was in this solution that the spectra of DHT treated histidine and tyrosine had been examined (6, 7). However, phosphorylase b in 1 M $KHCO_3$ at pH values of 8.4, 8.6 and 8.8 was found to be inactive. Earlier publications indicated that borate could be used in place of $KHCO_3$ (9, 10, 13). Therefore, this buffer was used when phosphorylase was treated with DHT at alkaline pH.

For work at acidic pH, three buffer systems were examined. Solutions of phosphorylase b in 20 mM GP, 20 mM ME, 1.5 mM EDTA, pH 6.1, in 20 mM GP, 1.5 mM EDTA, pH 6.1, and in 50 mM P_i, 1.5 mM EDTA, pH 6.1, all at 0°C, were treated with DHT. The results, shown in Table I, indicate that the reactivity of DHT with phosphorylase b was greatest in phosphate, less in glycerophosphate and least in glycerophosphate plus mercaptoethanol.

In order to elucidate the manner in which the buffer protected the enzyme, 20 mM GP, 20 mM ME, 1.5 mM EDTA, at pH 6.8 and 0°C, was made 1.28 mM with respect to DHT. After 30 minutes the reaction mixture was brought to room temperature. Absorption spectra of the sample revealed a 320 nm peak indicative of the reaction product between a sulfhydryl group and DHT. Hence, the protection of this

TABLE I

Effect of DHT upon Phosphorylase b
in Three Different Buffers

Buffer	Enz. conc. mg/ml	% Residual Activity 0 time	1 hour
20 mM GP, 1.5 mM EDTA pH 6.1	8.7	30.4	27
20 mM GP, 20 mM ME, 1.5 mM EDTA pH 6.1	9.7	111	96.6
50 mM P_i, 1.5 mM EDTA pH 6.1	8.7	6.5	0

Phosphorylase b passed through G-25 Sephadex column into respective buffers. Each sample at 0°C was treated with 0.51 mM DHT.

buffering system is due to the removal of DHT by a reaction with mercaptoethanol. On the other hand, the 20 mM GP, 1.5 mM EDTA, pH 6.1, buffer affords protection to the enzyme without directly reacting with DHT, as will be shown below.

Previous results demonstrated a rapid rate of hydrolysis of DHT in $KHCO_3$ at pH 8.8. Therefore, the rate of hydrolysis in borate, phosphate and glycerophosphate was investigated, in order to ensure that some DHT remained unhydrolyzed.

DHT was added to a series of seven buffers, as indicated in Figure 3. Aliquots were withdrawn at different time intervals and DHT content assayed for with N-acetyl-tyrosine. The results, shown in Figure 3, indicate that increasing temperature and pH increase the rate of hydrolysis. Also, it appears that DHT does not react with EDTA at a significant rate, since the presence or absence of EDTA did not affect the rate of DHT disappearance.

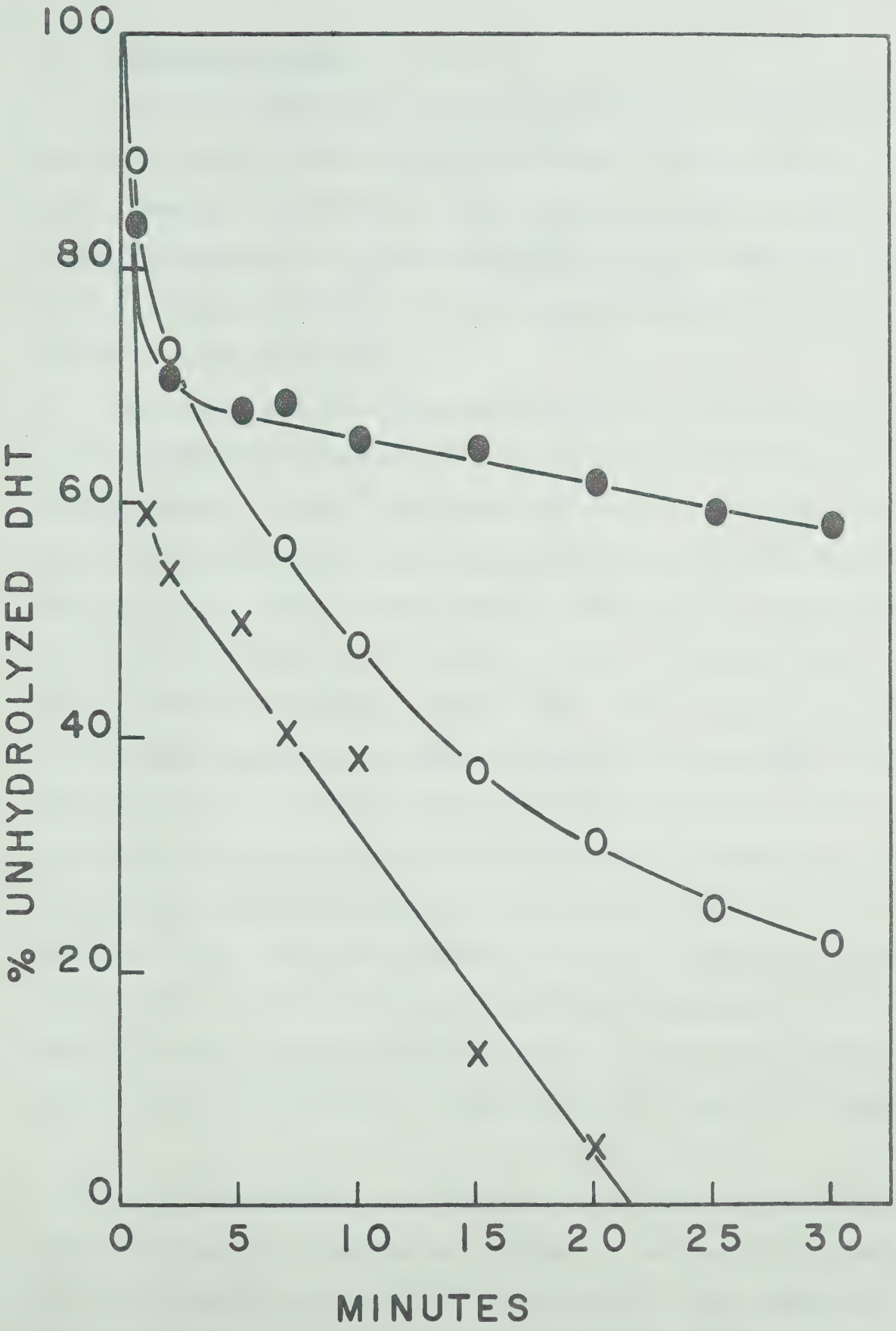
Figure 3

Hydrolysis of DHT. 31.8 mM DHT was diluted 10X into 80 mM borate, plus and minus 1.5 mM EDTA, pH 8.8, and 0.67 M $KHCO_3$, pH 8.8, all at 0°C. Likewise 31 mM DHT was diluted 10X into 50 mM P_i, plus and minus 4 mM EDTA, pH 6.1, and 20 mM GP, 4 mM EDTA, pH 6.1, all at 0°C and 50 mM P_i, 4 mM EDTA, pH 6.1, at room temperature. Aliquots were withdrawn at different times from each reaction mixture and added to a cuvette containing N-acetyltyrosine in 0.67 M $KHCO_3$, pH 8.8. After 30 minutes at room temperature the absorbance at 478 nm was obtained. This value was divided by absorbance expected if no DHT hydrolyzed. This value, presented as a percentage of hydrolysis, was plotted against the time at which the sample was removed from the reaction mixture. Zero time absorbance was calculated from the known DHT concentration in the reaction mixture.

O-O 3.18 mM DHT in 0.603 M $KHCO_3$, pH 8.8, at 0°C, or in 72 mM borate, plus or minus 3.6 mM EDTA, pH 8.8, at 0°C.

●-● 3.1 mM DHT in 45 mM P_i plus or minus 3.6 mM EDTA, pH 6.1, or 15 mM GP, 3.6 mM EDTA, pH 6.1, all at 0°C.

X-X 3.1 mM DHT in 45 mM P_i, 3.6 mM EDTA, pH 6.1, at room temperature.


100
80
60
40
20
0
% UNHYDROLYZED DHT
0
5
10
15
20
25
30
MINUTES

C. Amino Acid Study

DHT may react with tyrosine, histidine, lysine, arginine, serine, threonine and cysteine residues in proteins (7, 13, 16). However, spectral work has only been done on DHT reaction products of N-acetyltyrosine and N-acetylhistidine, in 1 M $KHCO_3$, pH 8.8 (7). These spectra were not examined below 350 nm.

DHT treatment of phosphorylase a or b in 50 mM P_i, 1.5 mM EDTA, pH 6.1, generated a major absorbance peak at approximately 320 nm. The modified protein retained this absorbance peak after dialysis into 20 mM GP, 5 mM ME, 1.5 mM EDTA, pH 6.8. Since these results could not be interpreted in terms of the previous spectral data, a complete DHT model compound study was carried out. The N-acetyl derivatives of tyrosine, histidine, lysine, arginine, tryptophan, serine and cysteine in 50 mM P_i, pH 6.1, were treated with DHT. In order to assure that the reaction went to completion and only monoazo products were formed, special conditions were employed, i.e., 0°C and 100-fold excess of the N-acetylamino acid to DHT. The reaction products were diluted into 0.67 M $KHCO_3$, pH 8.8, and 20 mM GP, 5 mM ME, 1.5 mM EDTA, pH 6.8, and the spectra obtained. The results are shown in Table II.

DHT disappearance was monitored by removing an aliquot from the reaction mixture and adding it to N-acetyltyrosine in 0.67 M $KHCO_3$, pH 8.8. If all the DHT in the reaction mixture disappeared within 30 minutes, it was assumed that

TABLE II

Molar Absorptivity Values for Reaction Products of DHT and N-Acetylamino Acids

DHT-treated N-acetyl-Amino Acids	pH	Wavelength of maximal absorbance (nm)	ε
N-acetyl-tyrosine	8.8	478	5.1×10^3
	8.8	320	1.53×10^4
	6.8	383	6.3×10^3
	6.8	320	9.5×10^4
N-acetyl-histidine	8.8	357	1.35×10^4
	6.8	350	1.03×10^4
N-acetyl-tryptophan	8.8	317	1.36×10^3
	6.8	313	2.13×10^3
N-acetyl-cysteine	8.8 or 6.8	318	8.4×10^3
		370	7.28×10^2
N-acetyl-lysine	8.8 or 6.8	no peak above 300 nm	-
N-acetyl-arginine	8.8 or 6.8	314	-
N-acetyl-serine	8.8 or 6.8	about 312	-

it had done so by a stoichiometric reaction with the N-acetylamino acid, since it was known that DHT would not completely hydrolyze within this time. In these cases, molar absorptivity values were calculated. However, in the reaction flasks containing N-acetylarginine and N-acetylserine, DHT was still present after 2 hours. In this situation some DHT had hydrolyzed, some had reacted with the N-acetylamino acid, and some remained unhydrolyzed. Since the percentage of each could not be determined, molar absorptivity values could not be calculated.

Part II. Inactivation Studies

A. Inactivation Studies on Phosphorylase b

Since the rate of reaction between DHT and amino acid residues in a protein was expected to show a pH dependence, phosphorylase b samples were treated with DHT within a pH range of 6.1 to 8.8, as shown in Table III. The loss of activity in acid solutions was as large as that in the alkaline solutions. Therefore, further experiments were carried out at neutral to acid pH, where it was hoped that DHT modification of the protein would be limited, but loss of activity would be large.

Phosphorylase b at 4.5 mg/ml in 50 mM P_i, 1.5 mM EDTA, pH 6.8 at room temperature was titrated with DHT. The per cent residual activity was determined at 10 minutes as shown in Figure 4. The reaction was stopped at 30 minutes and dialyzed into 20 mM GP, 5 mM ME, 1.5 mM EDTA, pH 6.8. Absorption spectra, shown in Figure 5, were obtained on some of the samples. DHT treatment of the protein resulted in a loss of activity with a corresponding linear increase in the absorbance at 320 nm as indicated in Figure 6. The linear correlation indicates that the increasing absorbance at 320 nm is caused by the same DHT modified residues as are responsible for activity loss. The absorption spectra show that these are not tyrosine or histidine residues.[1]

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[1]Further evidence that these residues are not modified by DHT treatment of phosphorylase b at pH 6.1 is presented in Part I

TABLE III

DHT Treatment of Phosphorylase b at Different pH Values

Phosphate Buffer				
DHT Conc mM	pH	% Residual Activity 0'	30'	60'
0.132	6.0	58	62	56
0.132	7.0	72	79	81
0.132	8.0	70	78	81
0.66	6.0	7	9	-
0.66	7.0	8	10	-
0.66	8.0	9	9	-

Borate Buffer				
DHT Conc mM	pH	% Residual Activity 0'	30'	60'
0.132	8.4	62	70	63
0.132	8.8	81	69	58
0.66	8.4	6	3	-
0.66	8.8	13	3	-

5.5 mg/ml phosphorylase b samples, either in 50 mM P_i, 1.5 mM EDTA, pH 6.0, 7.0, 8.0 or in 80 mM borate, 1.5 mM EDTA, pH 8.4, 8.8, all at room temperature, were treated with DHT.

Figure 4

DHT titration of phosphorylase b at room temperature. Phosphorylase b, 4.5 mg/ml, in 50 mM P_i, 1.5 mM EDTA, pH 6.8, at room temperature, was titrated with DHT. Activity was determined after 10 minutes of reaction.

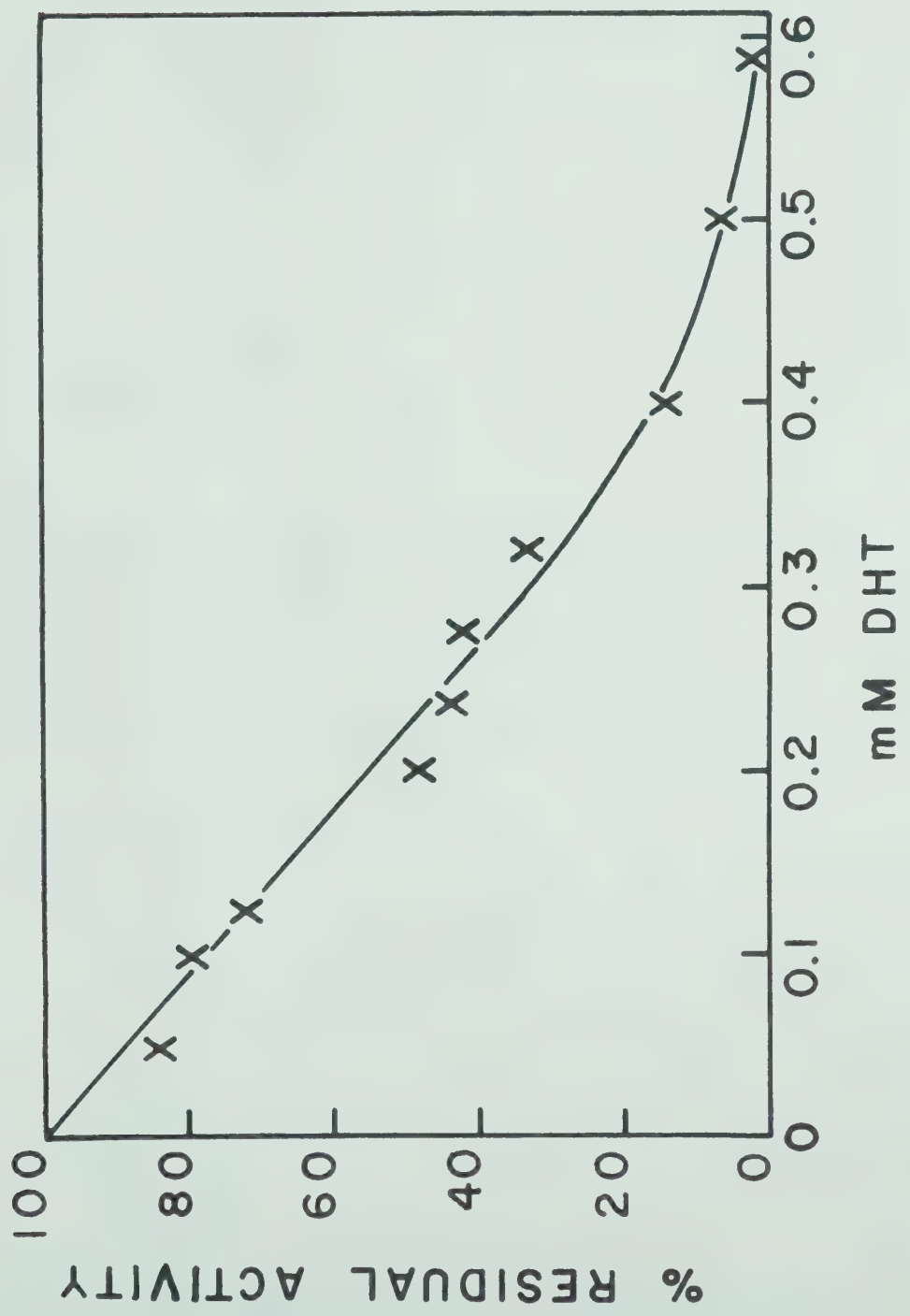

% RESIDUAL ACTIVITY
100
80
60
40
20
0
0
0.1
0.2
0.3
0.4
0.5
0.6
mM DHT

Figure 5

DHT treated phosphorylase b spectra. Phosphorylase b in 50 mM P_i, 1.5 mM EDTA, pH 6.8, at room temperature, was treated with increasing concentrations of DHT; reaction was stopped at 30 minutes and then dialyzed into 20 mM GP, 5 mM ME, 1.5 mM EDTA, pH 6.8.

A: Phosphorylase b treated with 0.5 mM DHT and then dialyzed. Protein concentration 0.58 mg/ml.

B: Phosphorylase b treated with 0.24 mM DHT and then dialyzed. Protein concentration 0.53 mg/ml.

C: Phosphorylase b treated with 0.05 mM DHT, and then dialyzed. Protein concentration 0.51 mg/ml.

D: Phosphorylase b control, concentration 0.616 mg/ml.

Each spectrum was blanked against 20 mM GP, 5 mM ME, 1.5 mM EDTA, pH 6.8.

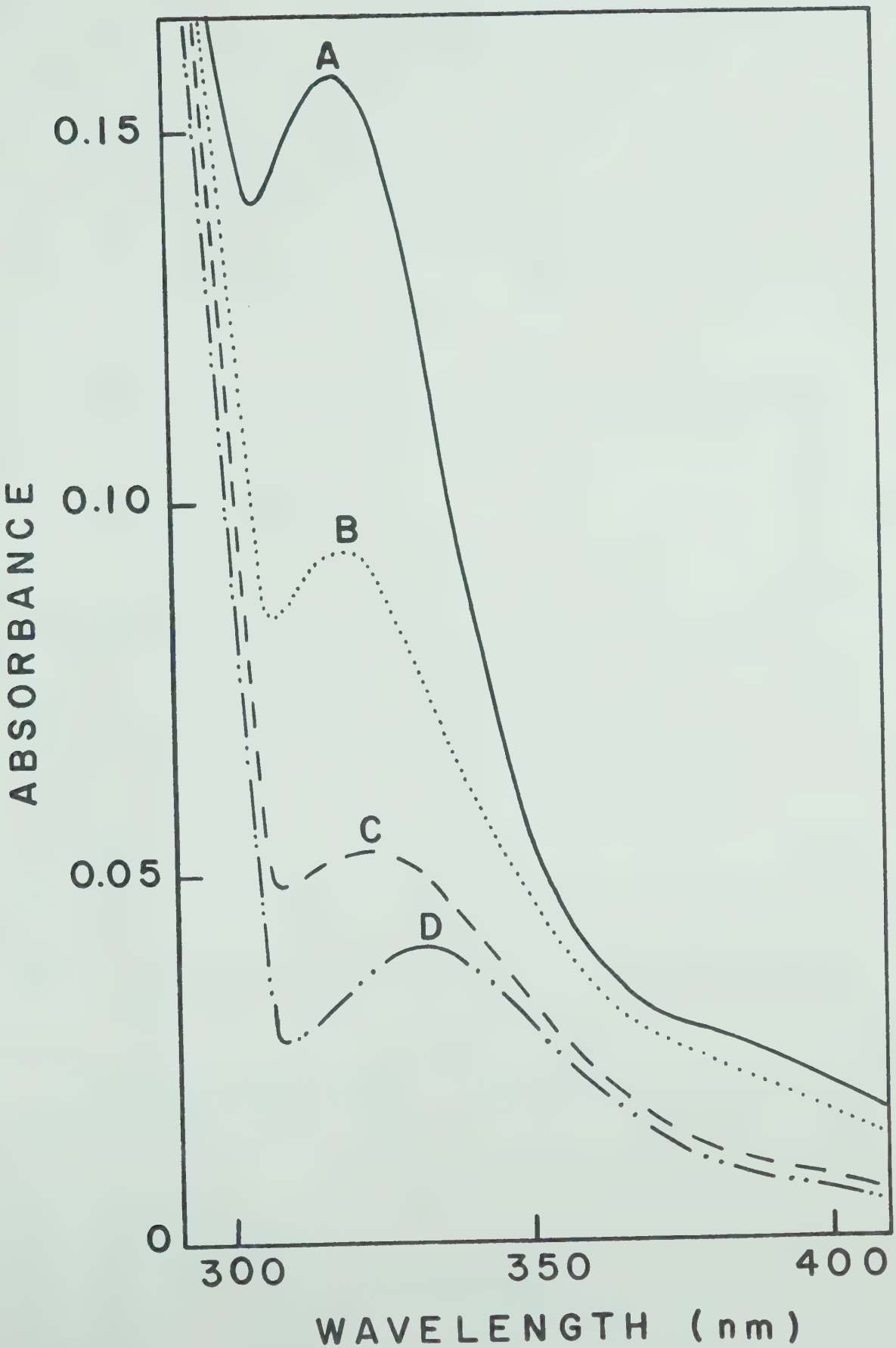
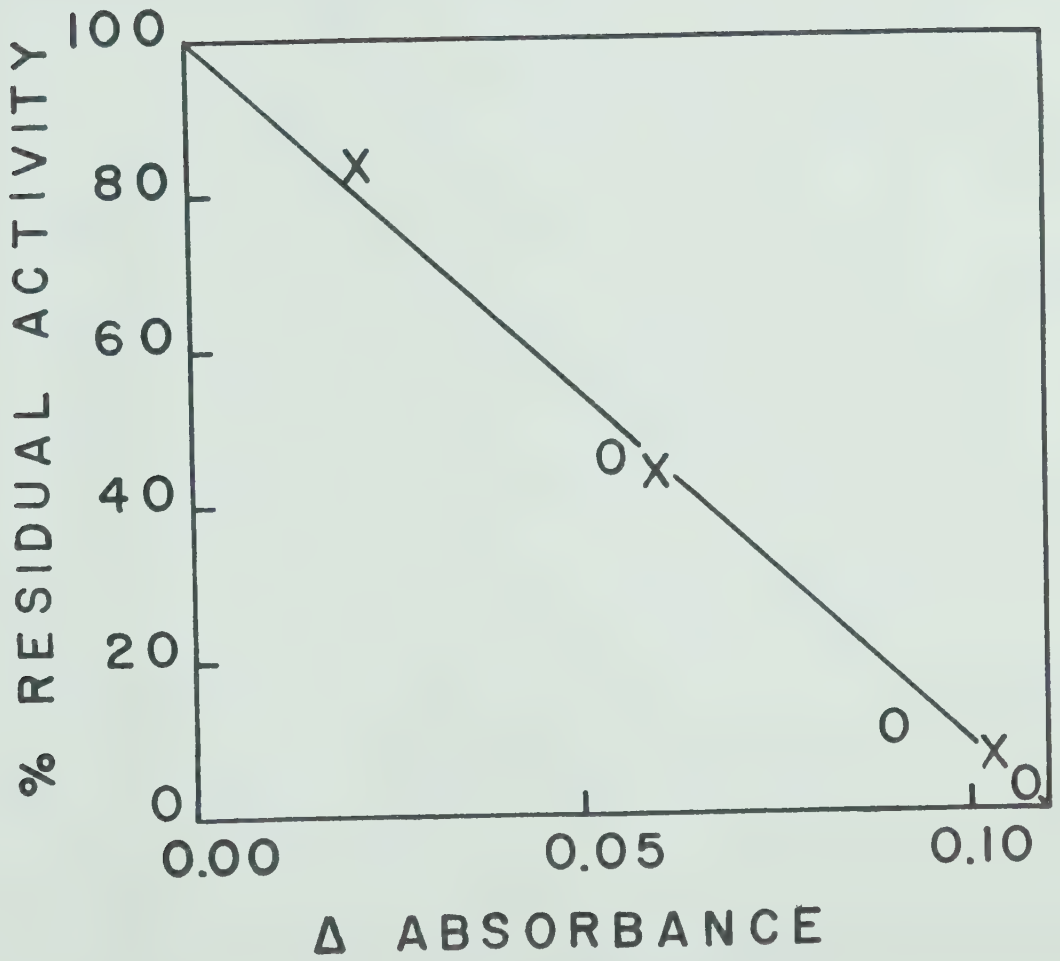

A
B
C
D
0.15
0.10
0.05
0
ABSORBANCE
300
350
400
WAVELENGTH (nm)

Figure 6

Increase in absorbance of DHT-treated phosphorylase a and b in relation to % residual activity. Absorbance values at 320 nm for DHT-treated phosphorylase b and 317 nm for DHT-treated phosphorylase a plus their respective controls were normalized to 0.51 mg/ml and 0.35 mg/ml respectively. From these normalized absorbance values the contribution by the control was subtracted. The resulting Δ absorbance was plotted against the corresponding % residual activity.

X-X Δ absorbance at 320 nm of DHT-treated phosphorylase b.

O-O Δ absorbance at 317 nm of DHT-treated phosphorylase a.


% RESIDUAL ACTIVITY
100
80
60
40
20
0
0.00
0.05
0.10
Δ ABSORBANCE

Another titration was carried out at a lower pH, to decrease protein reactivity, and at a lower temperature, to decrease the rate of DHT hydrolysis. Phosphorylase b at 5 mg/ml in 50 mM P_i, 1.5 mM EDTA, pH 6.1 at 0°C, was treated with DHT. The results are shown in Figure 7. Under these conditions, the DHT required to totally inactivate the enzyme is less than previously observed.

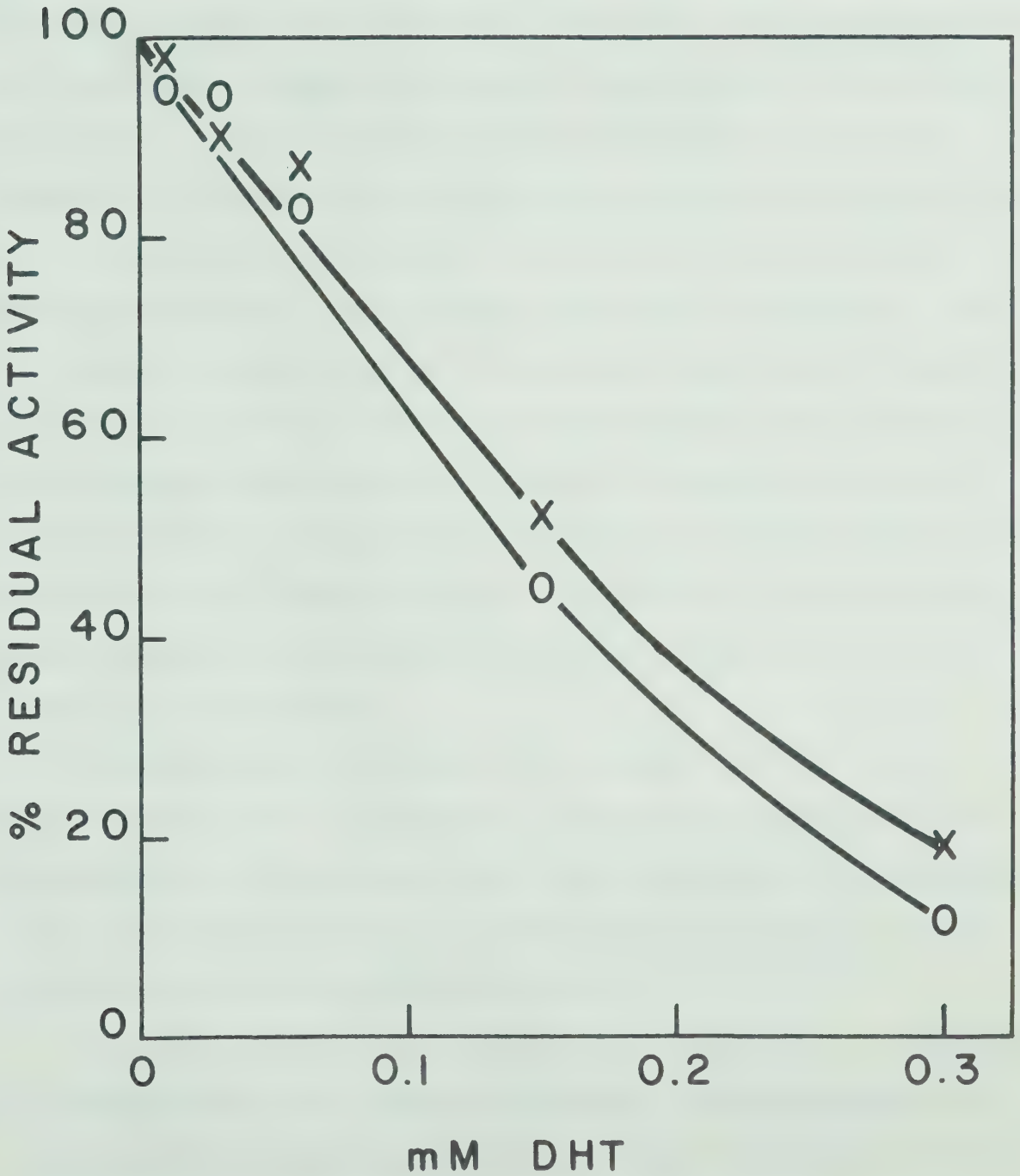
Figure 7

DHT titration of phosphorylase b at 0°C. Phosphorylase b, 5 mg/ml, in 50 mM P_i, 1.5 mM EDTA, pH 6.1, 0°C, was treated with increasing concentrations of DHT.

X-X % Residual activity at 0 minutes.

O-O % Residual activity at 30 minutes.

Note: "0 minute activity" refers to activity done within 0 to 30 seconds after addition of DHT to protein.


100
80
60
40
20
0
% RESIDUAL ACTIVITY
0
0.1
0.2
0.3
mM DHT

B. Rate Constant of Inactivation

It was hoped that the reaction between DHT and the protein could be slowed sufficiently to allow a rate constant of inactivation to be obtained. Lowering the temperature did not noticeably reduce the rate of inactivation. Therefore, the effect of varying protein and DHT concentrations was examined. Phosphorylase b at varying concentrations in 50 mM P_i, 1.5 mM EDTA, pH 6.1, 0°C, was treated with a constant DHT concentration. The results are shown in Figure 8. At 16.4 mg/ml the DHT does not inactivate the enzyme. Therefore, 1.43 moles of DHT per mole of enzyme monomer have reacted with residues which are not related to catalytic activity, such as the B_1 and B_2 sulfhydryl groups. Making appropriate corrections for these residues, one finds at 10 mg/ml that about 2 modified residues per monomer of enzyme result in 100% loss in activity.

The results of lowering both protein and DHT concentrations are shown in Figure 9. Making corrections in the same manner as above, one finds that treatment of the enzyme with 12 μM DHT results in an 80% inactivation due to modification of 0.8 residues per monomer.

In both titrations no substantial reduction in the extent of inactivation with time was observed, thus rendering calculation of the rate constant impossible. However, more of a time effect is noted with the lower enzyme concentration.

Figure 8

Phosphorylase b titration at constant DHT concentration. Varying concentrations of phosphorylase b in 50 mM P_i, 1.5 mM EDTA, pH 6.1, at 0°C, were treated with 0.254 mM DHT.

X-X % Residual activity at 0 minutes.

O-O % Residual activity at 30 minutes.

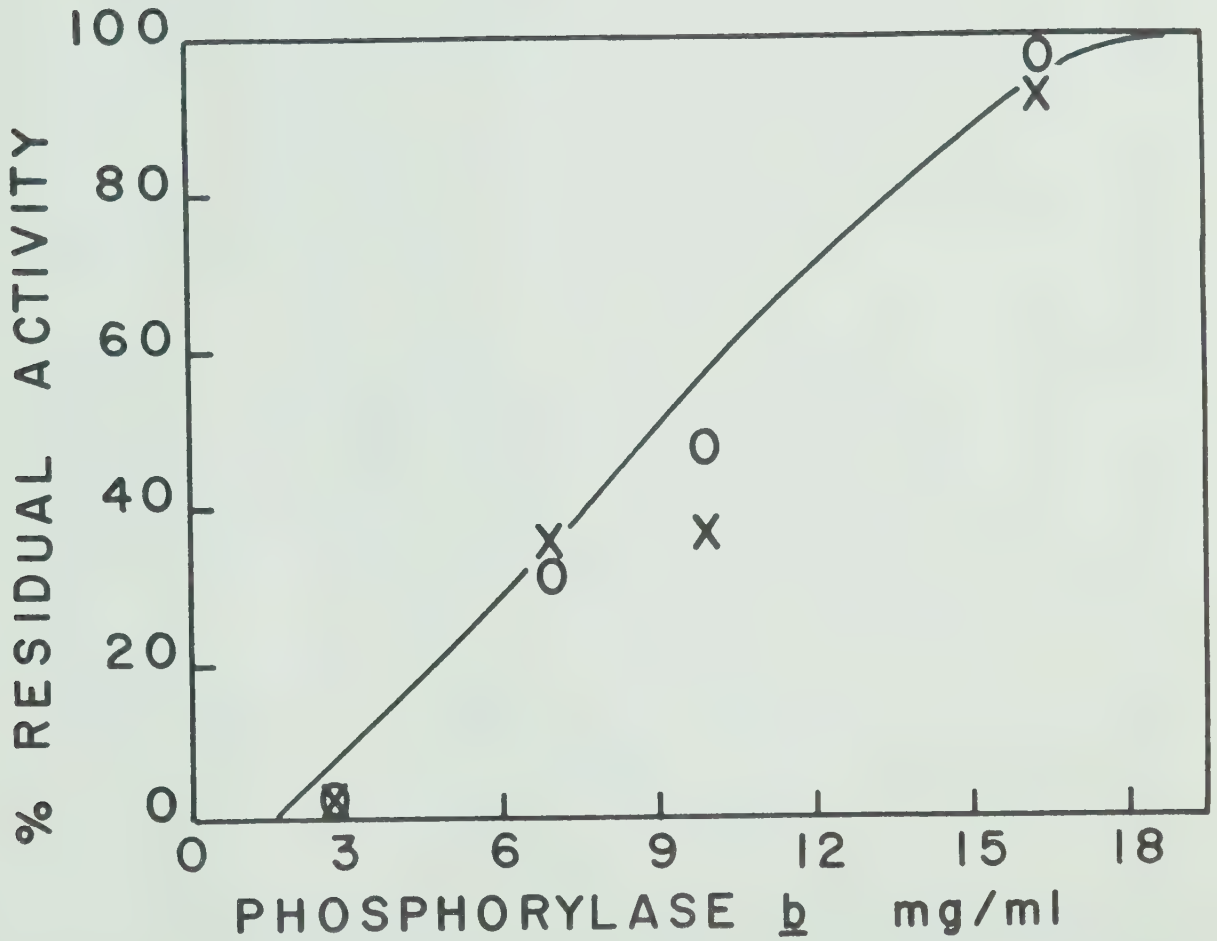
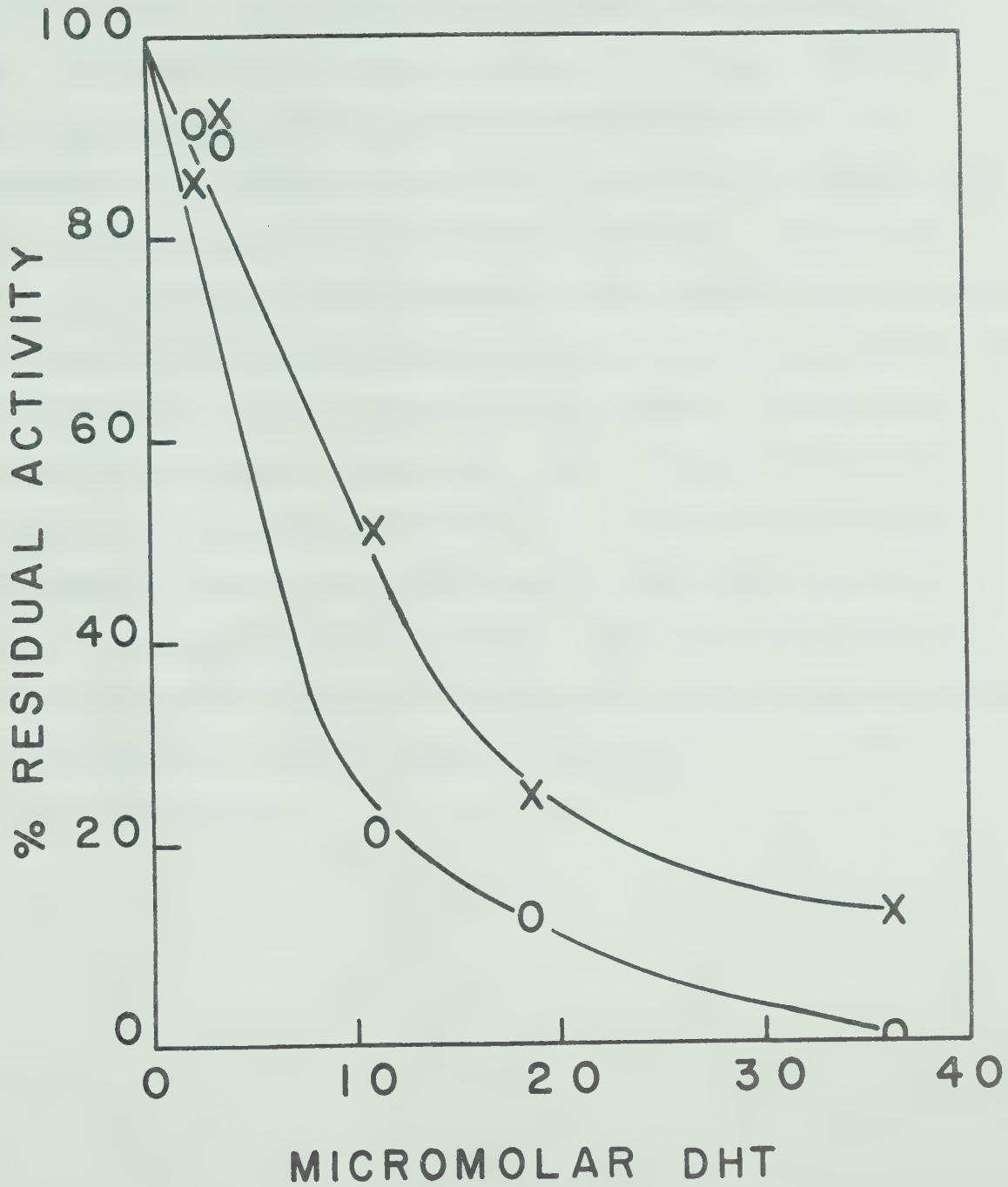

100
80
60
40
20
0
% RESIDUAL ACTIVITY
0
3
6
9
12
15
18
PHOSPHORYLASE b mg/ml

Figure 9

Titration of phosphorylase b at low enzyme and DHT concentration. Phosphorylase b, 0.5 mg/ml, in 50 mM P_i, 1.5 mM EDTA, pH 6.1, at 0°C, was treated with varying concentrations of DHT.

X-X % Residual activity at 0 minutes.

O-O % Residual activity at 30 minutes.


100
80
60
40
20
0
% RESIDUAL ACTIVITY
0
10
20
30
40
MICROMOLAR DHT

C. Titration of Phosphorylase a at pH 6.1

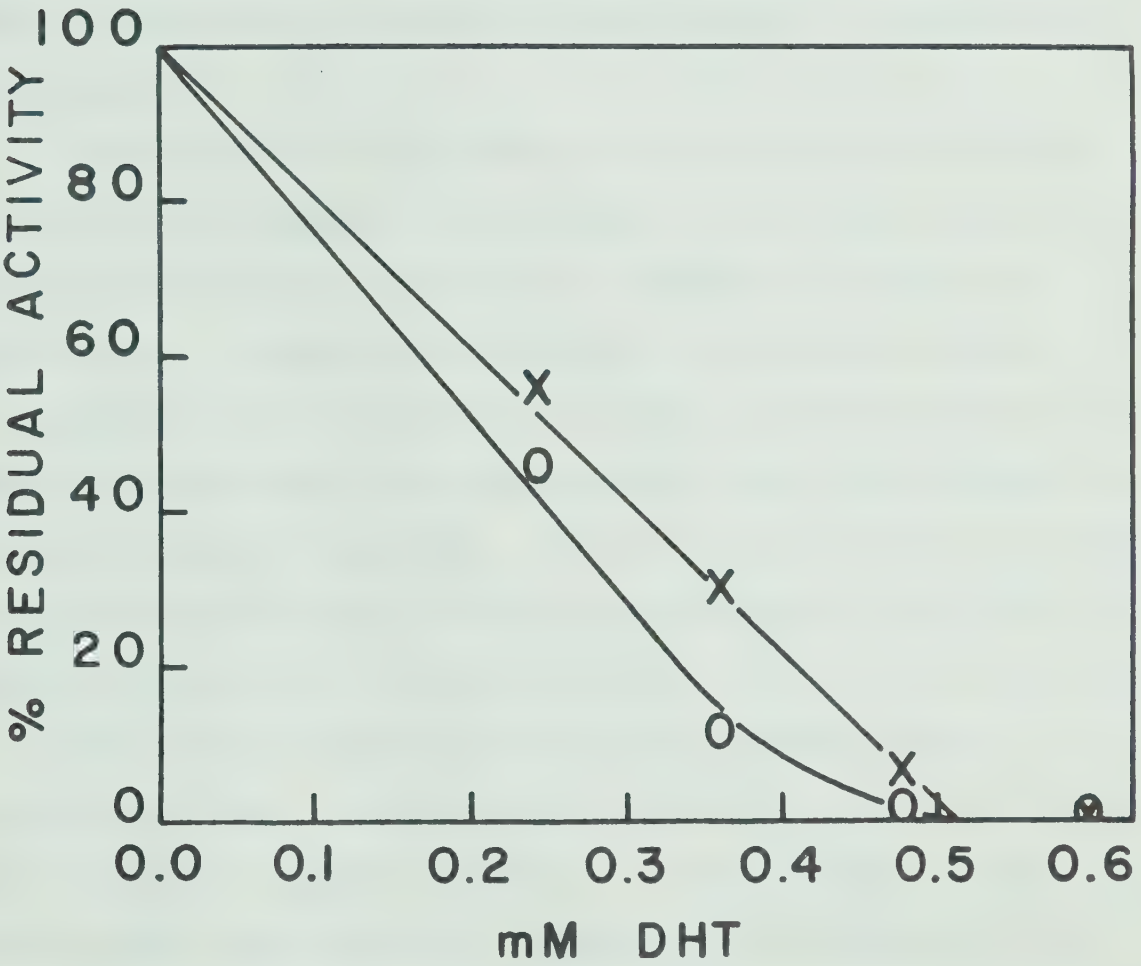
Phosphorylase a, 4.63 mg/ml in 50 mM P_i, 1.5 mM EDTA, pH 6.1 at 0°C, was treated with varying concentrations of DHT. The results are shown in Figure 10. About twice as much DHT was required to inactivate phosphorylase a as compared to b. After one hour the reaction was stopped, and each sample dialyzed into 20 mM GP, 5 mM ME, 1.5 mM EDTA, pH 6.8. The absorption spectra of these samples were similar to those of DHT treated phosphorylase b, showing a large peak at 317 nm and a small shoulder at 375-400 nm. There was a linear relationship between the loss in activity and the increase in absorbance at 317 nm, as indicated in Figure 6. The slope of this line is the same as that from the treatment of phosphorylase b with DHT. Therefore, DHT treatment of both proteins results in modification of the same residues, or residues with similar spectral properties, resulting in a loss of activity.

Figure 10

DHT titration of phosphorylase a at pH 6.1. Phosphorylase a, 4.36 mg/ml, in 50 mM P_i, 1.5 mM EDTA, pH 6.1, at 0°C, was treated with varying concentrations of DHT.

X-X % Residual activity at 0 minutes.

O-O % Residual activity at 60 minutes.


% RESIDUAL ACTIVITY
100
80
60
40
20
0
0.0
0.1
0.2
0.3
0.4
0.5
0.6
mM DHT

D. Titrations of Phosphorylase a and b at pH 8.8

Phosphorylases a and b were titrated with DHT in 80 mM borate, 1.5 mM EDTA, pH 8.8, at 0°C. Activities at 0 and 30 minutes were determined. The results at 30 minutes are shown in Figure 11. As with previous titrations, there was no substantial reduction in the extent of inactivation with time.

Between 60 and 90 minutes, the absorbance at 478 nm of each of the samples was measured. From this value the number of tyrosines modified per monomer of enzyme was calculated. Assuming one modified tyrosine per monomer is necessary to inactivate the enzyme, a prediction may be made as to residual activity expected, and this compared to observed residual activity. This is done in Table IV. The results indicate that in both phosphorylase a and b modification of tyrosine is not responsible for loss in activity.

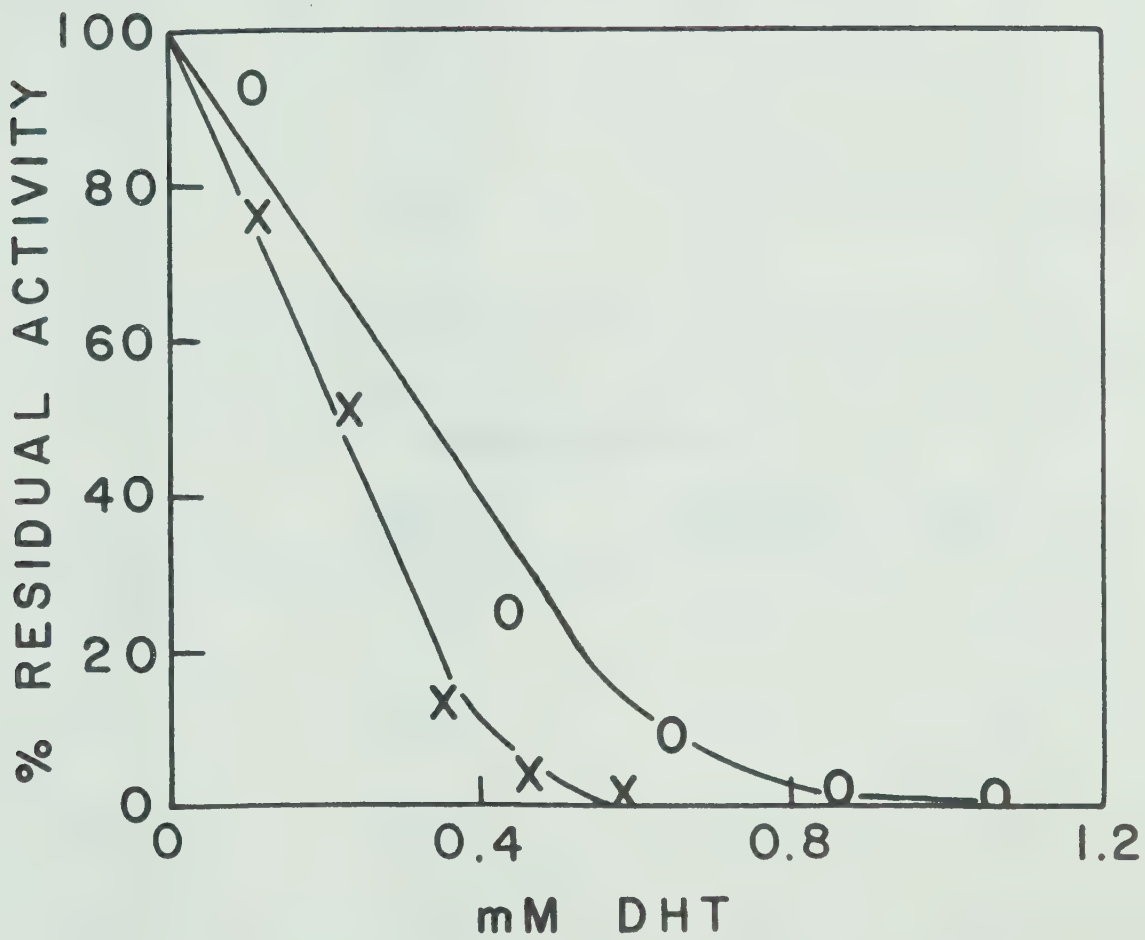
The results at pH 8.8 differ from those at pH 6.1 in significant ways. Tyrosine, and possibly some other groups not related to enzyme activity, are modified by DHT at pH 8.8, but not at pH 6.1. Because of this, more DHT is required at pH 8.8 to inactivate the proteins. Hence DHT is less selective at pH 8.8 than at pH 6.1 in attacking residues related to catalytic activity.

Figure 11

DHT titration of phosphorylase a and b at pH 8.8. Phosphorylase a, 4.25 mg/ml, and phosphorylase b, 4.24 mg/ml, both in 80 mM borate, 1.5 mM EDTA, pH 8.8, at 0°C, were treated with varying concentrations of DHT. Activities were determined after 30 minutes of reaction.

X-X Phosphorylase b.

O-O Phosphorylase a (assayed in the absence of AMP).


% RESIDUAL ACTIVITY
100
80
60
40
20
0
0
0.4
0.8
1.2
mM DHT

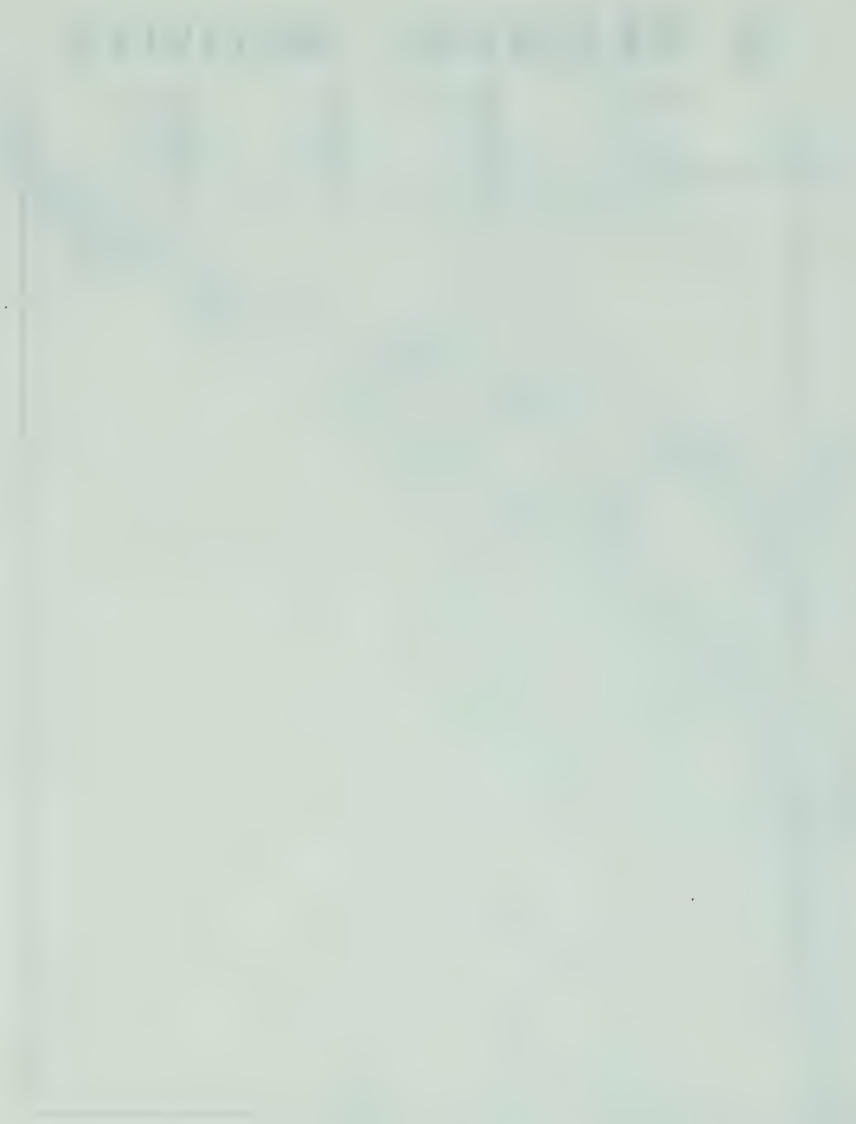


TABLE IV

Modification of Tyrosine in Phosphorylase a and b at pH 8.8

Phosphorylase b			
mM DHT	Tyrosine/ Monomer	% Residual Activity Predicted	Observed
0.117	0.17	83	76
0.234	0.21	79	51
0.351	0.28	72	14
0.467	0.38	62	4
0.587	0.51	49	2

Phosphorylase a			
mM DHT	Tyrosine/ Monomer	% Residual Activity Predicted	Observed
0.108	0.14	86	+ 98 - 93
0.43	0.49	52	+ 32 - 25
0.65	0.80	20	+ 15 - 10
0.86	0.93	8	+ 5 - 3
1.08	1.2	0	+ 2 - 2

Modified tyrosine per monomer of protein calculated from absorbance values at 478 nm of DHT treated phosphorylase a or b in 80 mM borate, 1.5 mM EDTA, pH 8.8, using $\varepsilon = 5.1 \times$

10^3. Predicted % residual activity was calculated assuming one modified tyrosine per enzyme monomer results in total inactivation. Observed % residual activity values are from 30 minute assay. Plus and minus refers to phosphorylase a assay in the present and absence of AMP.

Part III. Radioactive Titration

A. Quenching

The number of moles of reagent per mole of enzyme required for inactivation could be determined using radioactive DHT. Before commencing a radioactive DHT titration of the enzyme, it was necessary to select a scintillation solution and determine quenching, if any. In order to accomplish this the following experiments were done.

IAM phosphorylase b at 5 mg/ml, in 50 mM P_i, 1.5 mM EDTA, pH 6.1, 0°C, was made 0.4 mM with respect to radioactive DHT. Another portion of the same enzyme solution was made 0.396 mM with respect to non-radioactive DHT. Both were assayed after one and sixty minutes, and the reactions stopped at sixty minutes. Therefore, each reaction mixture contained the same percentage of hydrolyzed DHT, DHT-treated IAM phosphorylase b and DHT buffer reaction products. The radioactive DHT-treated IAM phosphorylase b had a residual activity of 10% at one minute and 7% after sixty minutes. The non-radioactive sample had 12% residual activity at one minute and 6% at sixty minutes.

Standard aliquots of the reaction mixture were counted in Bray's D scintillation solution with increasing amounts of untreated phosphorylase b present. The results are shown in Table V. Neither increasing the protein concentration, nor the presence or absence of buffer, quenched the number of counts, or affected the external standard.

Next, increasing concentrations of reaction mixture

TABLE V

Quenching by Phosphorylase b

#	Phosphorylase b mg per vial Untreated	Total	cpm	External Standard
1	0	0.232	18,816±94	1.022
2	0.050	0.282	18,764±131	0.998
3	0.100	0.332	18,602±130	1.000
4	0.200	0.432	18,779±132	0.990
5	0.502	0.734	18,857±132	0.998
6	1.00	1.236	18,637±130	0.995
7	0	0.232	18,800±132	0.981

Each scintillation vial contained 0.05 ml of the radioactive reaction mixture (i.e., 0.232 mg of treated protein). Samples 2 - 6 received 0.1 ml aliquots of increasing amounts of untreated IAM phosphorylase b in 50 mM P_i, 1.5 mM EDTA, pH 6.1, as indicated. Sample 1 contained no untreated protein. Sample 7 was the same as sample 1 except a 0.1 ml aliquot of 50 mM P_i, 1.5 mM EDTA, pH 6.1, was added.

were counted. The results, shown in Table VI, again indicated no quenching or significant change in external standard.

In both of these experiments a precipitate formed, the quantity of which was dependent upon protein concentration and time. Such precipitation should result in quenching. In order to investigate this, another scintillation solution, Aquasol, was used in which the protein was more soluble. Increasing amounts of the reaction mixture were added to Aquasol, and the results are shown in Table VI. C.p.m. observed in Aquasol and in Bray's D scintillation solutions were the same, thus indicating that protein precipitation had no quenching effect on the radioactivity.

The radioactive DHT stock solution was diluted into distilled water to a final concentration of 0.4 mM. This was the same concentration of the DHT in the original reaction mixture. Increasing amounts of the 0.4 mM radioactive DHT in distilled water were added to Bray's D scintillation solution and counted. The results are shown in Table VII. Once again it was demonstrated that the presence of buffer and protein did not quench the radioactivity.

Increasing volumes of the non-radioactive reaction mixture were added to a radioactive standard. The results are shown in Table VIII. As in the previous case, no quenching of radioactivity or significant change in the external standard was observed.

After removal of aliquots for the above experiments, both samples were dialyzed into 20 mM GP, 5 mM ME, 1.5 mM

TABLE VI

Quenching by Reaction Mixture

#	mg of DHT treated phosphor-ylase b	cpm Observed in Bray's D	External Standard	Normalized cpm in Bray's D	cpm Observed in Aquasol	External Standard
1	0.046	3,765±57	0.993	3,765±57	3,742±56	1.097
2	0.129	11,257±114	0.989	3,785±38	11,343±79	1.108
3	0.185	15,125±106	1.003	3,781±27	15,000±71	1.065
4	0.279	22,814±160	1.019	3,802±27	22,649±159	1.066
5	0.370	29,749±208	1.007	3,719±26	-	-
6	0.417	32,680±183	1.003	3,631±20	32,632±163	1.130
7	0.463	36,494±183	0.982	3,649±18	36,675±183	1.134
8	0.926	73,432±367	0.964	3,672±18	73,143±366	1.023
9	1.39	112,115±336	0.982	3,737±11	-	-

In samples 1 - 6, appropriate dilutions from the radioactive reaction mixture were made into distilled water, and then 0.1 ml aliquots added to the scintillation vials. In samples 7 - 9 aliquots were taken directly from the reaction mixture. Radioactivity was counted in both Aquasol and Bray's D scintillation solutions. The "normalized c.p.m. in Bray's D" values were obtained by dividing the multiple of protein concentration into the observed c.p.m.

TABLE VII

Quenching by Buffer and Protein

#	Column I DHT Diluted into H_2O, cpm	Column II DHT-Treated Phosphorylase *b* from Reaction Mixture, cpm
1	3,614±54	3,765±57
2	10,990±77	11,357±114
3	14,413±101	15,125±106
4	22,665±103	22,814±160
5	29 760±179	29,749±208
6	32,814±164	32,680±183

Results in Column II were compiled from Table VI. Results in Column I were obtained by the same dilutions into distilled water as used in obtaining results for Table VI. However, the starting mixture for these dilutions, unlike the previous case, was not a reaction mixture of 0.4 mM DHT, phosphorylase *b* and buffer, but rather 0.4 mM DHT in distilled water.

TABLE VIII

Quenching of Radioactive Standard by the Reaction Mixture

#	mg per vial	cpm	External Standard
1	0.046	12,595±88	0.999
2	0.092	12,660±89	1.005
3	0.231	12,666±89	0.972
4	0.462	12,636±89	0.976
5	0.924	12,636±88	0.970
6	Standard	12,567±88	1.017

For samples 1 - 3 appropriate dilutions from the non-radioactive reaction mixture were made into distilled water, and then 0.1 ml aliquots added to the scintillation vials. Samples 4 and 5 received aliquots taken directly from the reaction mixture. Each sample received a 0.1 ml aliquot of radioactive 5AT in distilled water as a standard.

EDTA, pH 6.8. After dialysis increasing amounts of the radioactive DHT treated IAM phosphorylase b were added to both Bray's D and Aquasol scintillation solutions. The results are shown in Table IX. In both cases the c.p.m. under similar conditions were the same. There was no quenching observed due to increasing amounts of DHT-treated IAM phosphorylase b. Both external standard and ratio (C^{14}/H^3) were found not to be related to c.p.m.

Increasing amounts of the dialyzed non-radioactive DHT-treated IAM phosphorylase b were added to scintillation vials containing a ^{14}C-n-hexadecane standard. Samples were counted in both Bray's D and Aquasol scintillation solutions. The results are shown in Table X. As before, they indicate that DHT-treated IAM phosphorylase b does not quench, and no correlation between c.p.m. and external standard was observed.

Finally the efficiency of the Beckman LS-200 was determined for both scintillation solutions using ^{14}C-n-hexadecane as the standard. C.p.m. were found to be 94% of the d.p.m. in Bray's D scintillation solution and 92% in Aquasol scintillation solution.

In the following experiments radioactivity was counted in Bray's D scintillation solution. Results obtained from samples taken before and after dialysis were used without correction. However, each time samples were counted, the efficiency of the machine was determined to ensure that it remained constant.

TABLE IX

Quenching by DHT-Treated IAM Phosphorylase b

#	mg per vial	cpm in Bray's D	External Standard	Ratio C^{14}/H^3	Normalized cpm in Bray's D
1	0.045	867±26	0.976	2.420	867±26
2	0.122	2,540±76	0.984	2.529	931±28
3	0.188	3,898±58	0.990	2.558	975±15
4	0.267	5,742±57	0.977	2.437	957±10
5	0.356	7,903±79	1.000	2.500	991±10
6	0.445	9,606±67	0.982	2.362	961±7
7	0.890	19,393±97	0.987	2.406	970±5
8	4.45	93,888±282	0.857	2.027	939±3

The concentration of radioactive DHT treated IAM phosphorylase b after dialysis was 4.45 mg/ml. For samples 1 - 5, appropriate dilutions were made into 20 mM GP, 5 mM ME, 1.5 mM EDTA, pH 6.8, then 0.1 ml aliquots were added to each scintillation vial. Aliquots for samples 6 - 8 were removed directly from the protein solution. These samples were counted in Bray's D and Aquasol scintillation solutions. "Normalized c.p.m." were obtained as explained in Table VI. 24 hours after preparation the samples were counted again, this time with the ratio of C^{14} to H^3 instead of external standard. The c.p.m. observed with the second counting were the same as the first, hence the ratio is listed above beside the exact value of the first counting.

TABLE X

Quenching of ^{14}C-n-Hexadecane by DHT-Treated IAM Phosphorylase b

#	mg per vial	Bray's D cpm	External Standard	Aquasol cpm	External Standard
1	0.048	17,057±52	0.964	16,557±83	1.069
2	0.095	17,027±52	0.959	16,580±83	1.098
3	0.143	17,029±52	0.954	16,521±83	1.071
4	0.190	17,023±52	0.933	16,553±83	1.084
5	0.285	17,035±52	0.959	16,498±83	1.069
6	0.380	17,001±52	0.939	16,562±83	1.061
7	0.475	17,057±52	0.971	16,554±83	1.085
8	0.950	16,999±52	0.946	16,590±83	1.074
9	1.900	16,982±52	0.970	-	-
Standard		17,060±52	0.938	16,642±83	1.097

Each sample received 0.1 ml of ^{14}C-n-hexadecane dissolved in the respective scintillation solution. The concentration of the non-radioactive DHT-treated IAM phosphorylase b after dialysis was 4.75 mg/ml. For samples 1 - 6 appropriate dilutions were made into distilled water, then 0.1 ml aliquots were added to each scintillation vial. Samples 7 - 9 received aliquots directly from the protein solution.

B. Preliminary Inactivation Studies with Radioactive DHT

Phosphorylase b, IAM phosphorylase b (sometimes in the presence of substrates) and apo IAM phosphorylase b, in 50 mM P_i, 0.1 mM EDTA, pH 6.1, 0°C, were treated with DHT. At the same time activities were determined, aliquots were removed and diluted 2X into 50 mM GP, 50 mM ME, pH 6.8. These samples were then dialyzed into 20 mM GP, 10 mM ME, 2 mM EDTA, pH 6.8. Using these dialyzed samples, the DHT incorporation was calculated. The results are shown in Table XI.

Phosphorylase prepared in DTT had incorporation values lower than phosphorylase prepared in ME. The reason for this is not understood. However, the results show that 0.82 modified residues result in 19% residual activity, indicating that one modified residue per enzyme monomer results in total inactivation. Subsequent increasing incorporation did not lower the per cent residual activity to zero, thus indicating that residues not related to catalytic activity were modified. Since the other enzyme forms show the same per cent residual activity, but even higher incorporation values, a similar pattern of inactivation is suggested.

IAM treated and native phosphorylase b both had the same incorporation values, indicating that the B_1 and B_2 sulfhydryl groups do not react with DHT. IAM treated apo phosphorylase b also showed similar values, indicating that the removal of PLP does not quantitatively alter incorporation. Except for AMP, the substrates did not protect the enzyme. The protection afforded by AMP was limited.

TABLE XI

Incorporation Values for DHT-Treated Phosphorylase b, IAM Phosphorylase b and Apo IAM Phosphorylase b

Enzyme Sample	Enz Conc mg/ml	mM DHT	Time (min)	DHT Incorporation/ Monomer	% Residual Activity
IAM Phosphorylase b	5	0.35	0	1.52	18
			5	2.36	9
			10	2.78	8
			15	2.50	8
IAM Phosphorylase b*	5	0.35	0	0.82	19
			30	1.65	11
Phosphorylase b	5	0.35	0	1.4	19
			30	2.4	7
IAM Phosphorylase b plus 2 mM AMP	5	0.35	0	1.43	34
			30	2.37	18
IAM Phosphorylase b plus 10 mM G-1-P	5	0.35	0	1.43	14
			30	2.37	19
IAM Phosphorylase b plus 1% glycogen	3.8	0.41	0	1.48	13
			30	2.86	2
Apo IAM Phosphorylase b	5	0.35	0	2.23	-
			30	2.67	-

*Indicates phosphorylase b from preparation using DTT, not ME.

C. Effect on DHT Incorporation of pH and of Reduction of the Pyridoxal-5'-Phosphate Linkage

Reduced IAM phosphorylase b and IAM phosphorylase b, both in 80 mM borate, 1.5 mM EDTA, pH 8.8, and in 50 mM P_i, 1.5 mM EDTA, pH 6.1, all at 0°C, were treated with radioactive DHT. After one minute the reaction was stopped, and the activity determined. The contents of the reaction flask were dialyzed into 20 mM GP, 5 mM ME, 1.5 mM EDTA, pH 6.8. However, a sample from the IAM phosphorylase b, pH 6.1, reaction flask was also passed through a G-25 Sephadex column into 20 mM GP, 5 mM ME, 1.5 mM EDTA, pH 6.8. Absorption spectra were obtained of the dialyzed samples and activity and DHT incorporation values were obtained on all samples. The results are shown in Table XII. They demonstrate that modification of 1.0 residues per enzyme monomer at pH 8.8, or 0.77 residues per enzyme monomer at pH 6.1, results in total inactivation. Therefore, only residues essential to catalytic activity are modified within the first minute of reaction. Previous results indicated that allowing the reaction to proceed for longer periods of time would not substantially increase the loss in activity. This in turn must mean that the remaining unreacted DHT modifies other residues not related to catalytic activity.

The spectral results indicate that the groups modified at pH 6.1 are different from those modified at pH 8.8. Reduction of the protein does not influence DHT modification of the enzyme at either pH. Dialysis causes the

TABLE XII

Spectral Properties, DHT Incorporation Values and % Residual Activity of DHT-Treated Proteins

Enzyme Form	mg/ml in Rx	% Residual Activity 1 min	% Residual Activity After Dialysis	Molecules of DHT Incorporated /Monomer	Molar Absorptivity Values 320 nm	Molar Absorptivity Values 387 nm
IAM Phosphorylase b, pH 6.1	6.29	28	26	0.56	1.18×10^4	5.0×10^3
IAM Phosphorylase b, pH 8.8	6.93	77	89	0.15	2.44×10^4	1.17×10^4
Reduced IAM Phosphorylase b, pH 6.1	7.47	4	11	1.05	1.09×10^4	2.31×10^3
Reduced IAM Phosphorylase b, pH 8.8	6.47	69	86	0.16	3.04×10^4	1.19×10^3
IAM Phosphorylase b, pH 6.1, passed through G-25 column	6.29	28	23	0.51	-	-

The different enzyme forms at 0°C in 50 mM P_i, 1.5 mM EDTA, pH 6.1, or in 80 mM borate, 1.5 mM EDTA, pH 8.8, were treated with 0.252 mM DHT. After one minute the reaction was stopped and the enzymic activity determined. The first four samples listed above were dialyzed into 20 mM GP, 5 mM ME, 1.5 mM EDTA, pH 6.8; the last sample was passed through a G-25 Sephadex column into the same buffer. Molar absorptivity values were calculated on the basis of the DHT incorporated after correcting for the absorbance of the control.

activity of the protein treated at pH 8.8 to increase, but the activity of the protein treated at pH 6.1 remains the same. Passage of DHT treated protein through a column of G-25 Sephadex instead of dialysis does not affect incorporation values.

DHT modified lysine, histidine, tyrosine, and to some extent arginine, are destroyed by acid hydrolysis (16). Therefore, an amino acid analysis was obtained of the DHT treated protein with the highest incorporation value. The results shown in Table XIII demonstrate that no significant changes can be observed within the limits of error of this type of analysis.

TABLE XIII

Amino Acid Analysis Carried Out on a Sample of Reduced IAM Phosphorylase b Treated with DHT at pH 6.1

Amino Acid	# per dimer Theoretical Value	24 hr	48 hr	72 hr
Lysine	86	84	85	86
Histidine	38	40	41	43
Arginine	118	112	110	114
Aspartic Acid	182	184	180	185
Threonine	64	66	66	66
Serine	54	52	48	45
Glutamic Acid	188	183	185	186
Glycine	92	93	91	93
Alanine	120	Standard		
Valine	114	111	117	125
Methionine	40	39	36	8
Isoleucine	90	81	84	90
Leucine	150	149	151	150
Tyrosine	68	67	69	37
Phenylalanine	72	72	77	74

D. Effect of pH on DHT Incorporation and Stability

One portion of an IAM phosphorylase b sample was passed through a G-25 Sephadex column into 50 mM P_i, 1.5 mM EDTA, pH 6.1. The protein in this buffer at a concentration of 8.0 mg/ml at 0°C was treated with 0.512 mM DHT. The other portion of the IAM phosphorylase b sample was passed through a similar column into 80 mM borate, 1.5 mM EDTA, pH 8.8. In this buffer at a concentration of 7.9 mg/ml at 0°C, the protein was treated with 1.02 mM DHT. After one minute both reactions were terminated. The sample at pH 6.1 showed 9% residual activity, while the pH 8.8 sample had 22% residual activity.

Addition of the GP, ME buffer at pH 7.0 to the reaction mixture at pH 6.1, raised the pH to 6.5. This solution was separated into two samples, A and B. Sample A was passed through a G-25 Sephadex column into 50 mM P_i, 1.5 mM EDTA, pH 6.1. B was passed through a similar column into 20 mM GP, 5 mM ME, 1.5 mM EDTA, pH 6.8. Examination of these samples revealed that A had 15% residual activity with 1.22 moles of DHT incorporated per mole of enzyme monomer, while B had 12% residual activity with 1.65 moles of DHT incorporated per mole of enzyme monomer.

The addition of GP, ME buffer at pH 7.0 to the reaction mixture at pH 8.8 resulted in lowering the pH to a value of 8.5. One portion was adjusted to pH 8.8 and 30 minutes after the addition of DHT, the absorbance at 478 nm was measured. From this value it was calculated that 0.54 tyrosines per monomer of enzyme were modified. The reaction mixture at pH 8.5

was separated into two samples, C and D. Sample C was passed through a G-25 Sephadex column into 80 mM borate, 1.5 mM EDTA, pH 8.8, while sample D was passed through a similar column into 20 mM GP, 5 mM ME, 1.5 mM EDTA, pH 6.8. Examination revealed that sample C retained 39% residual activity while incorporating 2.21 moles of DHT per mole of monomer of the enzyme. Sample D had 44% residual activity with 2.75 moles of DHT incorporated per mole of monomer of the enzyme.

These results indicate that the isotope incorporated into the protein is stable with respect to the pH changes encountered during dialysis.

Previous results indicated that DHT treatment of the protein for a limited time at pH 8.8 would inactivate the protein by modifying one residue per enzyme monomer. However, these conclusions were based upon a protein sample which was not completely inactivated. The above results show that when the DHT concentration is raised, in order to obtain more inactivation, substantial modification of residues not essential to catalytic activity occurs, even with a limited reaction time. One of these residues is tyrosine, the nature of the others is not known.

DHT treatment of the protein at pH 6.1 instead of pH 8.8 results in considerably less modification of residues not related to catalytic activity. Therefore, when it is desired to inactivate the enzyme by modification of residues related to catalytic activity, the reaction should be carried out at pH 6.1.

E. Radioactive Titration

Solutions of phosphorylase b, at concentrations of 5 mg/ml and 0.5 mg/ml in 50 mM P_i, 1.5 mM EDTA, pH 6.1, 0°C, were treated with varying concentrations of radioactive DHT. Activities were measured at 0 minutes and 30 minutes. At these same times, aliquots from the reaction mixtures were diluted 2X into 20 mM GP, 20 mM ME, 1.5 mM EDTA, pH 6.8 and then dialyzed into 20 mM GP, 5 mM ME, 1.5 mM EDTA, pH 6.8, and DHT incorporation determined. The moles of DHT incorporated per mole of monomer of phosphorylase b was plotted against the per cent residual activity determined before dialysis. The results are shown in Figures 12 and 13. They indicate that modification of one residue per enzyme monomer produces total inactivation.

The results also show the importance of enzyme concentration and the length of time the reaction is allowed to proceed. At 5 mg/ml modification of the residue essential for catalytic activity takes place immediately. Allowing the reaction to proceed for a longer period of time only results in modification of residues not essential for catalytic activity. Hence, incorporation values increase with time, but percent residual activity remains the same.

The results at 0.5 mg/ml are different. By lowering the protein concentration, the rate of inactivation is reduced enough so that the residue essential for catalytic activity is not completely modified within the first few seconds. Hence, incorporation values increase, and percent residual activity values decrease, with time.

Figure 12

Radioactive DHT titration of phosphorylase b. Phosphorylase b at a concentration of 5 mg/ml in 50 mM P_i, 1.5 mM EDTA, pH 6.1, at 0°C, was titrated with ^{14}C-DHT at concentrations of 0.036 mM, 0.06 mM, 0.08 mM, 0.12 mM, 0.15 mM, 0.20 mM and 0.30 mM, for the points from left to right.

X-X % Residual activity at 0 minutes.

O-O % Residual activity at 30 minutes.

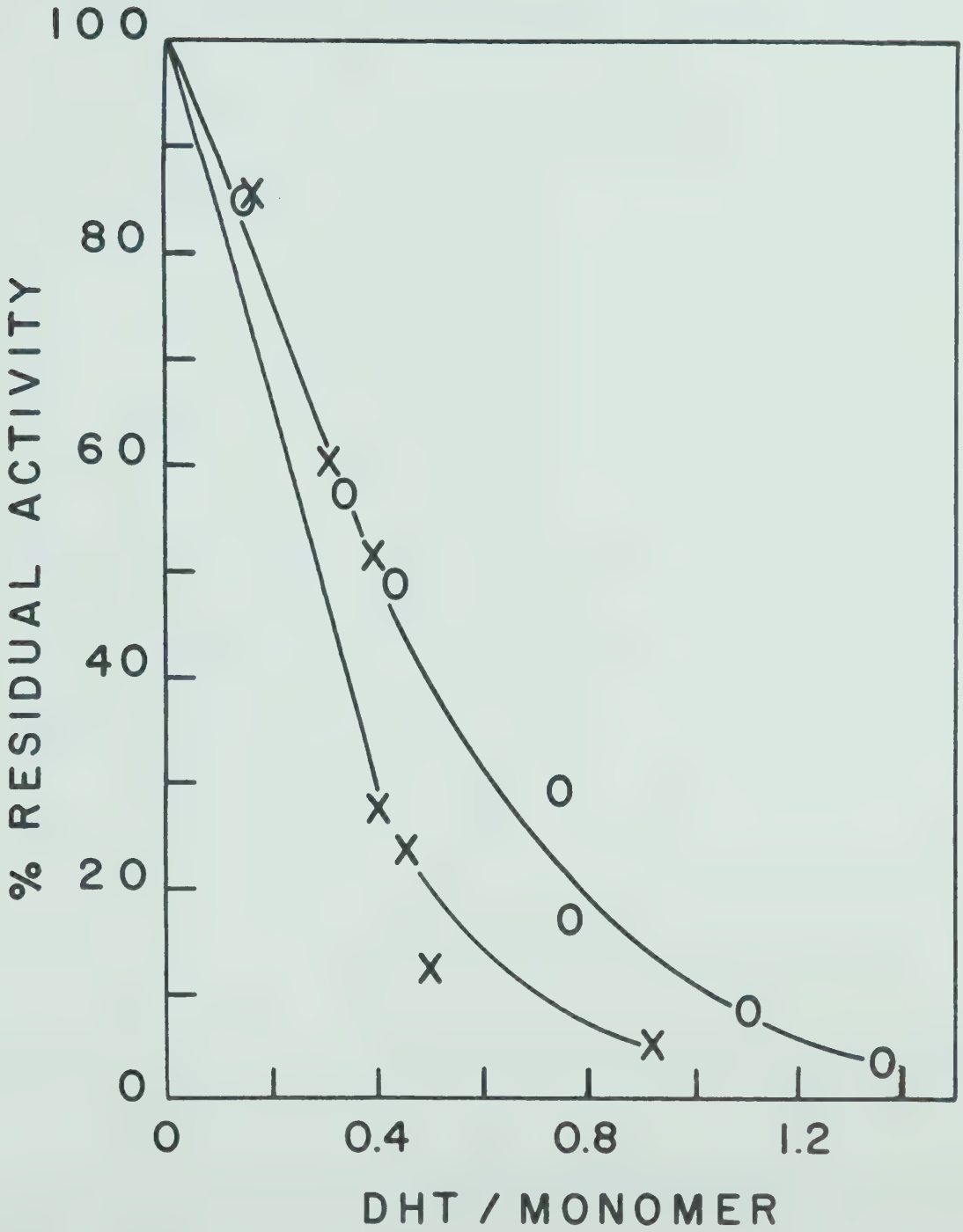
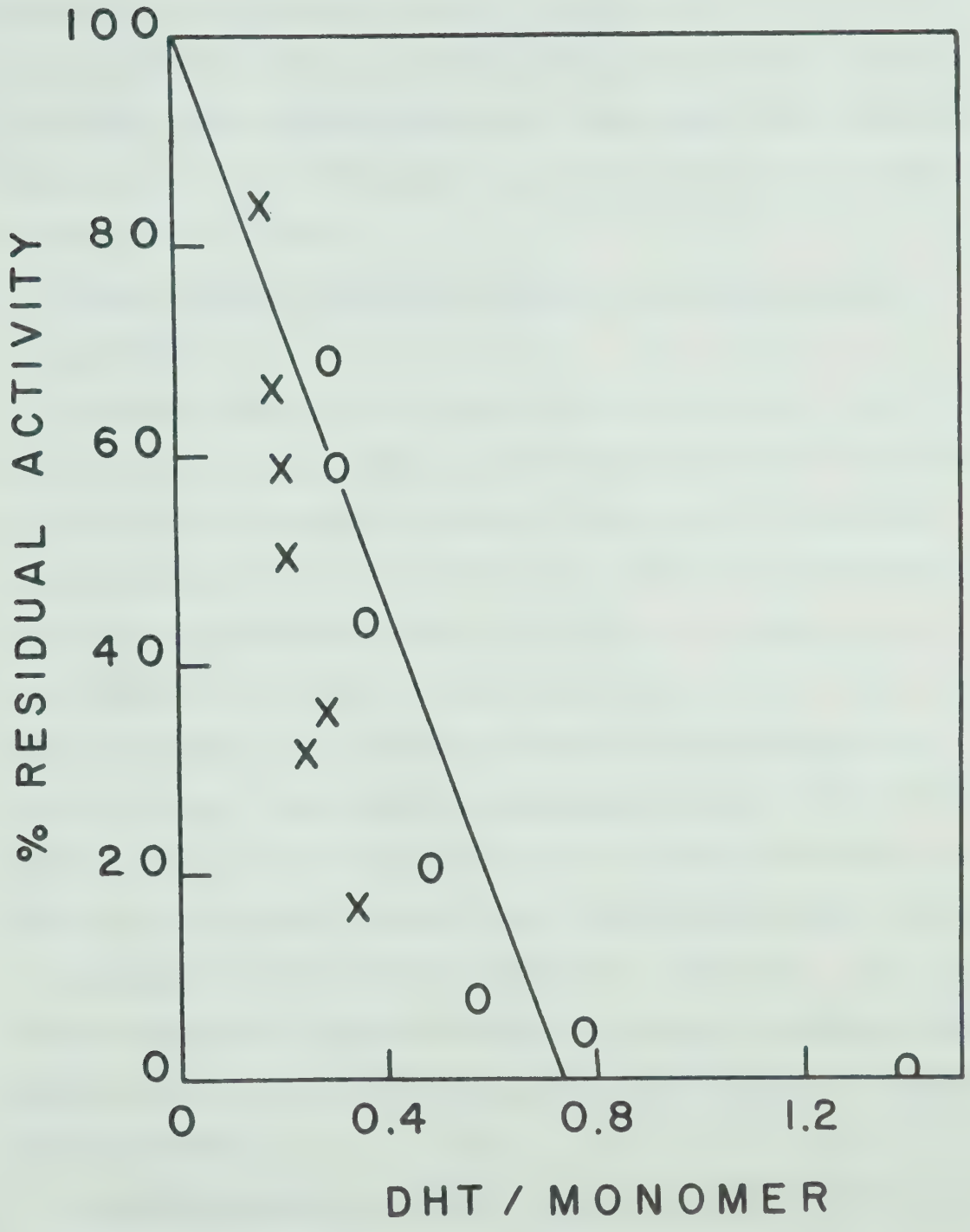

100
80
60
40
20
0
% RESIDUAL ACTIVITY
0
0.4
0.8
1.2
DHT / MONOMER

Figure 13

Radioactive DHT titration of phosphorylase b. Phosphorylase b at a concentration of 0.5 mg/ml in 50 mM P_i, 1.5 mM EDTA, pH 6.1, at 0°C, was titrated with ^{14}C-DHT at concentrations of 2.2 µM, 6.7 µM, 8.7 µM, 12 µM, 15 µM, 18.4 µM and 30 µM for the points from left to right.

X-X % Residual activity at 0 minutes.

O-O % Residual activity at 30 minutes.


100
80
60
40
20
0
% RESIDUAL ACTIVITY
0
0.4
0.8
1.2
DHT / MONOMER

Part IV. Phosphorylase b Reactivity with DHT

The radioactive results presented in the previous section indicated that phosphorylase b treated with DHT in 50 mM P_i, 1.5 mM EDTA, pH 6.1, contained approximately one modified group per enzyme monomer and was completely inactive. The purpose of the following experiments was to elucidate the nature of this group.

A. Reactivity of Sulfhydryl Groups in Phosphorylase b with DHT

Shimada reported (13) that DHT reacted with a sulfhydryl group in myosin producing an increase in absorbance at 320 nm. Evidence presented in Part I, Section B, indicated that DHT reacts with the sulfhydryl group of mercaptoethanol producing an absorbance peak at 320 nm. The amino acid study presented in Part I, Section C, also shows that the reaction product of DHT with N-acetylcysteine produces a peak at 318 nm. DHT modified phosphorylase b in 20 mM GP, 5 mM ME, 1.5 mM EDTA, pH 6.8, shows an absorbance peak at 320 nm with a molar absorptivity value in agreement with that of monoazotetrazole-N-acetylcysteine. Therefore, the following experiment was carried out in order to examine the possible reaction between the sulfhydryl groups in phosphorylase b and DHT.

Phosphorylase b in 50 mM P_i, 1.5 mM EDTA, pH 6.1, at 11 mg/ml and 0°C, was treated with 0.808 mM DHT. A control sample was prepared under the same conditions, but was not treated with DHT. Samples at 0 time and 30 minutes were

removed for activity assays and ultracentrifuge studies. The residual activity for the treated protein was 15.7% at 0 time and 9.9% at 30 minutes. After 32 minutes the reaction was terminated. The control and DHT-treated protein samples were dialyzed into 1% GP, 1% KCl, 0.15 mM ME, 1.5 mM EDTA, pH 6.8. After dialysis sedimentation velocity studies were carried out on the samples which were removed from the reaction mixture at zero and 30 minutes. The results are shown in Table XIV. The phosphorylase b sample treated for 32 minutes with DHT, after dialysis into 1% GP, 1% KCl, 0.15 mM ME, 1.5 mM EDTA, pH 6.8, at a concentration of 9.2 mg/ml, was treated with 10 mM radioactive iodoacetamide at 30°C. The control sample (phosphorylase b not treated with DHT) in the same buffer at 9.6 mg/ml and 30°C was likewise treated with 10 mM radioactive IAM. Samples were taken from both the control and DHT reaction mixtures at 0 time, 2 hours, 4 hours and 6 hours for enzymic activities, peptide studies and incorporation studies. Likewise, samples were taken at 0 time and 2 hours for ultracentrifugation studies. These results are shown in Table XV.

The samples, removed for peptide analysis, were treated with pepsin as described in Materials and Methods. The peptides were separated on paper by high voltage electrophoresis at pH 6.5 for 45 minutes, and scanned for radioactivity. The strip scan results indicated that both DHT-treated and regular phosphorylase b contained reactive B_1, B_2, N and A sulfhydryl groups.

TABLE XIV

Effect of DHT Upon Sedimentation Velocity of Phosphorylase b

Enzyme	Time	% Residual Activity	S_{20}	% Area
Control	0'	100	7.8 12.2	98.5 1.5
DHT-treated phosphorylase b	0'	15.7	5.5 8.5 11.5	6.6 85.8 7.6
Control	30'	100	7.7 11.9	98.5 1.5
DHT-treated phosphorylase b	30'	9.9	5.6 9.1 15.2	20.5 66.2 13.3

% Residual activity of DHT-treated protein and control were determined from samples taken directly from the reaction mixture. Sedimentation velocity results were obtained on samples after dialysis.

TABLE XV

Effect of DHT Upon Titration of Sulfhydryl Groups by IAM

Enzyme	% Residual Activity	IAM Incorporation /Monomer	S_{20}	Area
DHT-IAM treated phosphorylase b at 0'	0.6	-	5.4 9.0 15.4	9.3 57.4 33.3
IAM treated control, 0'	99	-	-	-
DHT-IAM treated phosphorylase b at 2 hours	0.3	1.45	5.6 9.1 15.2	19.9 27.6 52.5
IAM treated control at 2 hours	73.6	1.57	-	-
DHT-IAM treated phosphorylase b at 4 hours	0.1	1.94	-	-
IAM treated control at 4 hours	65.0	2.1	-	-
IAM treated control at 6 hours	54.4	2.28	-	-

% Residual activity and ultracentrifugation results were determined on samples taken directly from the reaction mixture. Incorporation values were determined from dialyzed samples obtained in the following manner. Aliquots were removed from the reaction mixture at the indicated times, and diluted 2X in 50 mM GP, 50 mM ME, 1.5 mM EDTA, pH 6.8. These samples were then dialyzed into 20 mM GP, 20 mM ME, 1.5 mM EDTA, pH 6.8.

These results indicate that the DHT phosphorylase b with 10% residual activity is only 20% monomer. Therefore, the N and A sulfhydryl groups are not modified by DHT, since it is known that their modification results in inactivation and complete dissociation (31). Treatment of the DHT phosphorylase b with IAM produced an increase in the percentage of monomer. This indicates that the N and A sulfhydryl groups were titrated by IAM, and therefore could not have reacted with DHT. There was also an increase in the percentage of fast sedimenting material, which must represent a new aggregate state of the enzyme.

Incorporation of ^{14}C-IAM into both the control and DHT protein sample was identical. Therefore, the integrity of the sulfhydryl groups in DHT-treated protein is the same as in the native enzyme, and this serves as further evidence that DHT does not modify sulfhydryl residues. The results show that about 50% of the B_1 and B_2 sulfhydryl groups were reactive. As is often observed, the remaining percentage was oxidized under the severe dialysis conditions (34).

Analysis of the ^{14}C-IAM peptides revealed that there were reactive B_1, B_2, N and A sulfhydryl residues in both the control and DHT treated proteins. Hence, DHT treatment does not quantitatively titrate any one of these groups.

B. Spectral Study

In Part I spectra of certain monoazotetrazole-N-acetyl-amino acids were presented. With the knowledge of where the maxima occur and what their pH dependence is, the following experiment was carried out in the hope of characterizing the residue which reacts with DHT.

IAM phosphorylase b at a concentration of 10 mg/ml in 50 mM P_i, 1.5 mM EDTA, pH 6.1, at 0°C, was treated with 0.344 mM radioactive DHT. After one minute the reaction was stopped. An enzymic assay determined that the residual activity was 30.4%. The DHT-treated IAM phosphorylase b was passed through a G-25 Sephadex column into 20 mM GP, 20 mM ME, 1.5 mM EDTA, pH 6.8. Isotopic counting revealed that 0.92 moles of DHT were incorporated per mole of enzyme monomer. Ultracentrifugation studies indicated that, as in the previous example, the enzyme remained predominantly dimer. This result is shown in Figure 14. Ultraviolet spectra were obtained on this treated protein at pH values varying from 2.6 to 11.4. The spectra are shown in Figure 15. The protein showed a peak at 321 nm which did not vary in magnitude between pH 6.8 and 8.8. However, raising or lowering the pH,so that the enzyme was denatured, generated new spectra. At pH 11.4 the 321 nm peak disappeared, and a new peak at 388 nm appeared. At pH 2.6 the 321 nm peak was reduced.

There are two possible explanations for these results. First, the 321 nm and the 388 nm peaks could be due to the

Figure 14

Effect of DHT upon the structure of phosphorylase b. IAM phosphorylase b, at 7.3 mg/ml, contains 0.92 DHT modified residues per enzyme monomer. Pictures were taken at 0, 8, 16, 24 and 32 minutes. The S_{20} values were 8.6 for the fast component and 5.6 for the slow component. Their per cent areas were 93% and 7% respectively.

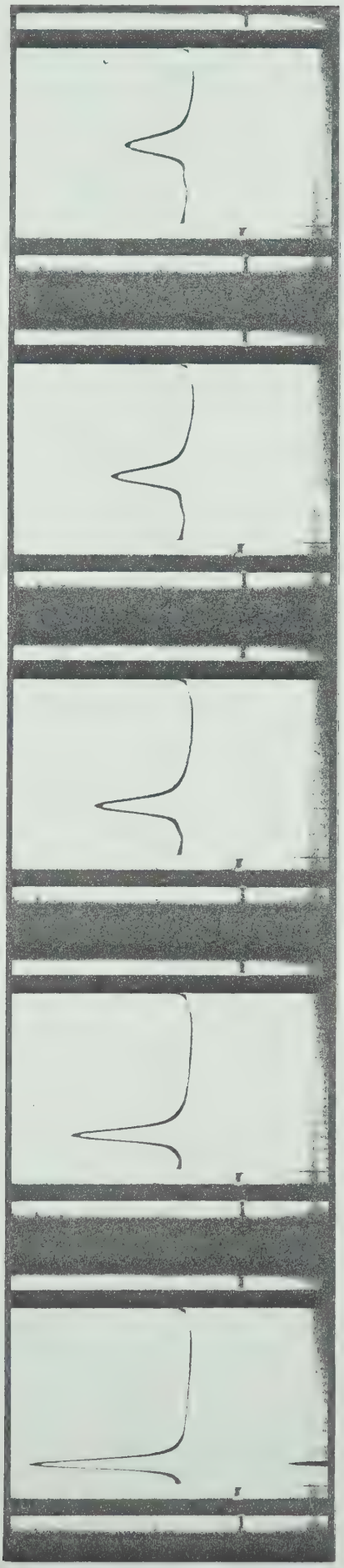


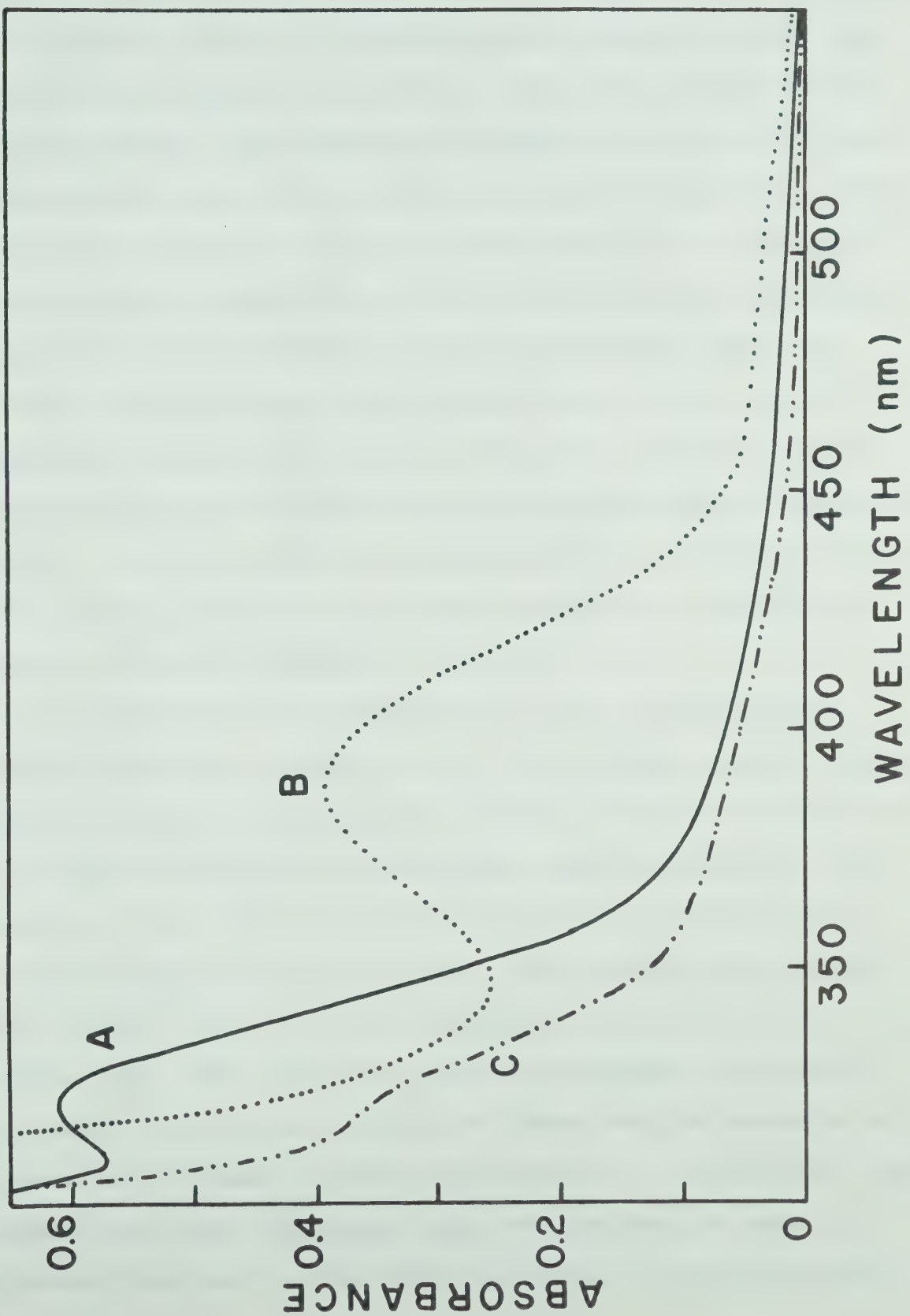
Figure 15

Absorption spectra of DHT-treated IAM phosphorylase b. This IAM phosphorylase b contains 0.92 DHT modified residues per enzyme monomer. 10 N NaOH or 90% formic acid was used to adjust pH. Each spectrum was blanked against 20 mM GP, 20 mM ME, 1.5 mM EDTA adjusted to the proper pH.

A: DHT-treated IAM phosphorylase b, 5.2 mg/ml, in 20 mM GP, 20 mM ME, 1.5 mM EDTA, pH 6.8, 7.3, 8.0, 8.8.

B: DHT-treated IAM phosphorylase b, 5.2 mg/ml, in 20 mM GP, 20 mM ME, 1.5 mM EDTA, pH 11.4.

C: DHT-treated IAM phosphorylase b, 5.2 mg/ml, in 20 mM GP, 20 mM ME, 1.5 mM EDTA, made 5% in formic acid, pH 2.3.


ABSORBANCE
0.6
0.4
0.2
0
350
400
450
500
WAVELENGTH (nm)
A
B
C

same chromophore. The shift could be caused by the denaturation of the protein at pH 11.4. This implies that the peak at 321 nm or 388 nm is produced by the reaction of DHT with a particular amino acid residue. Since monoazotetrazole-N-acetylhistidine has a maximum absorbance at 360 nm, it could be suggested that this was the amino acid in question. The second possibility is that the 388 nm peak is not caused by the reaction of DHT with any amino acid residue, but rather represents the absorbance of free PLP released when the protein was denatured. It is known that free PLP shows absorbance peaks at 328 nm and 388 nm at neutral pH. The former peak decreases and the latter increases with increasing pH(35). Also, 5.2 mg/ml of phosphorylase b at pH 12.2 will show a peak at 388 nm with a magnitude of 0.35 absorbance units, due to the release of PLP (36).

Experiments were conducted in order to distinguish between these two possibilities. The spectral samples containing the DHT-treated protein shown in Figure 15 were stored at 5°C for three weeks. Absorbance spectra obtained on the samples at pH 6.8 and 7.3 after this storage period still showed the major peak at 321 nm. Each sample was treated with 2% SDS, which is known to denature the protein and release PLP. The pH of both samples increased two tenths of a unit. Absorption spectra of these samples revealed a reduced 320 nm peak and the appearance of a 388 nm peak, thus establishing that the peaks were not one and the same. It appeared that part of the 320 nm and all of the 388 nm peak

were due to the release of PLP. Since the free cofactor was known to be pH sensitive, the SDS treated DHT protein at pH 7.5 was diluted 10X into 20 mM GP, 20 mM ME, 1.5 mM EDTA, so that the final pH values after dilution were 6.8, 8.1 and 8.9. Absorption spectra obtained on these samples showed that the 320 nm peak decreased and the 388 nm peak increased with increasing pH.

Therefore, the 321 nm peak disappearance and 388 nm peak appearance at pH 11.3 of DHT-treated IAM phosphorylase b is the result of the enzyme being denatured and releasing PLP. The nature of the 321 nm peak in the DHT protein between pH values of 6.8 and 8.8 is unclear. Obviously histidine and tyrosine cannot be involved since their reaction products would produce absorption peaks at 360 nm and 478 nm respectively. Lysine and tryptophan appear unlikely since the former would have no absorbance peak above 300 nm, and the absorbance peak of the latter should be pH sensitive. Therefore, either the cofactor or one of the other amino acid residues reacts with DHT.

C. PLP Studies

1. Loss of PLP from DHT-treated phosphorylase b

During the course of this study a number of results were obtained which indicated that the PLP in the protein was direclty or indirectly affected by DHT.

In Part II, Section A, results from the titration of phosphorylase b with DHT at room temperature were presented. Fluorescence spectra of the DHT treated samples indicated that, in comparison to the control, the 535 nm peak was reduced, either due to quenching or loss of PLP. In order to determine the magnitude of the latter, the samples were precipitated with perchlorate and the supernatant solution was examined at 290 nm to determine the concentration of free PLP. The results are shown in Table XVI. With an increasing loss in percent residual activity there was not a corresponding loss in PLP.

These results suggest that the PLP environment or the cofactor itself may be modified by DHT, but the resulting loss in activity is not wholly due to the removal of PLP from the enzyme.

2. DHT treatment of native, reduced and apophosphorylase b

Native, reduced and apophosphorylase b with controls in 50 mM P_i, 1.5 mM EDTA, pH 6.8, were treated with DHT at room temperature for 30 minutes. Each sample was dialyzed into 50 mM GP, 50 mM ME, 1.5 mM EDTA, pH 6.8. After dialysis samples were removed for ultracentrifugation and spectral studies. The activity and ultracentrifugation results are

TABLE XVI

Percentage PLP Removed from Phosphorylase b by Treatment with DHT

Sample No.	mM DHT conc. in Rx.	% Residual Activity Before Dialysis	% Loss of PLP determined after Dialysis
1	0.05	83.9	-
2	0.1	79.6	-
3	0.12	67.1	0
4	0.2	48.4	1.9
5	0.24	44.1	0.7
6	0.28	42.2	2.6
7	0.32	33.7	10.6
8	0.4	14.6	11.6
9	0.5	6.7	28.1
10	0.59	1.9	25.8
Control		100	3.2

Conditions for titration of phosphorylase b were presented in Figure 4. After dialysis samples were precipitated with 0.3 M perchlorate and the absorbance of the supernatant at 290 nm was determined. Using a molar absorptivity value of 6.5×10^3 for free PLP, the percentage released from the enzyme was calculated.

shown in Table XVII. The sedimentation results show that the inactive DHT-treated native and reduced protein remains predominantly dimer. The spectral results for DHT-treated native phosphorylase b showed the characteristic peak at 320 nm. The DHT-treated apoenzyme also showed a 320 nm peak while the control apophosphorylase b had no 320 nm peak. These spectra are shown in Figure 16. The appearance of a 320 nm peak after DHT treatment of phosphorylase b, whether or not PLP is present, indicates that DHT modifies an amino acid residue.

An attempt was made to reconstitute the DHT-treated apoenzyme, but this resulted only in a 7% return of activity (see Table XVII).

3. Loss of isotope and PLP from DHT-treated phosphorylase b

DHT-treated IAM phosphorylase b from the spectral experiment (Part IV, Section B) in 20 mM GP, 5 mM ME, 1.5 mM EDTA, pH 6.8, was stored at 5°C for one month. After this storage period the protein was dialyzed against 20 mM GP, 5 mM ME, 1.5 mM EDTA, pH 6.8. Absorption spectra of the dialyzed protein revealed that it retained its 321 nm peak. However, emission spectra showed that this protein contained no PLP and radioactive counting indicated that 52% of the isotope was lost. The protein retained 0.48 moles of DHT per mole of enzyme monomer. Therefore, storage of DHT-treated IAM phosphorylase b results in lability of the cofactor. Removal of the PLP is accompanied by loss of the isotope, thus suggesting that some of the DHT directly modifies the cofactor.

TABLE XVII

Sedimentation Velocity and % Residual Activity of DHT Treated Native, Apo and Reduced Phosphorylase b

Enzyme Form	Time Minutes	% Residual Activity	Sedimentation Velocity Results $S_{20,w}$	% Area
Native control	0	100		
	30	100	10.7	100
Native DHT	0	4		
	30	5	5.3	24.8
			7.7	75.2
Reduced control	0	64		
	30	53	8.5	100
Reduced DHT	0	5		
	30	7	6.5	23.6
			9.0	76.4
Apo control	0	2	6.9	39.9
			16.0	60.4
Apo DHT	0	0	5.7	23.6
			16.5	76.4
Apo reconstituted	-	60	-	-
Apo DHT reconstituted	-	7	-	-

Native, apo- and reduced phosphorylase b in 50 mM P_i, 1.5 mM EDTA, pH 6.8, at a concentration of 5.7, 6.5 and 6.6 mg/ml respectively were treated with 0.42 mM DHT at room temperature. Samples were removed from the reaction mixture

at 0 and 30 minutes to determine enzymatic activity. The remaining reaction mixture was dialyzed into 50 mM GP, 50 mM ME, 1.5 mM EDTA, pH 6.8. From the dialyzed proteins, samples were taken for sedimentation velocity studies. The dialyzed apo control and DHT-treated apo protein were used for reconstitution experiments. All % residual activity values were calculated based upon the native control phosphorylase b.

Figure 16

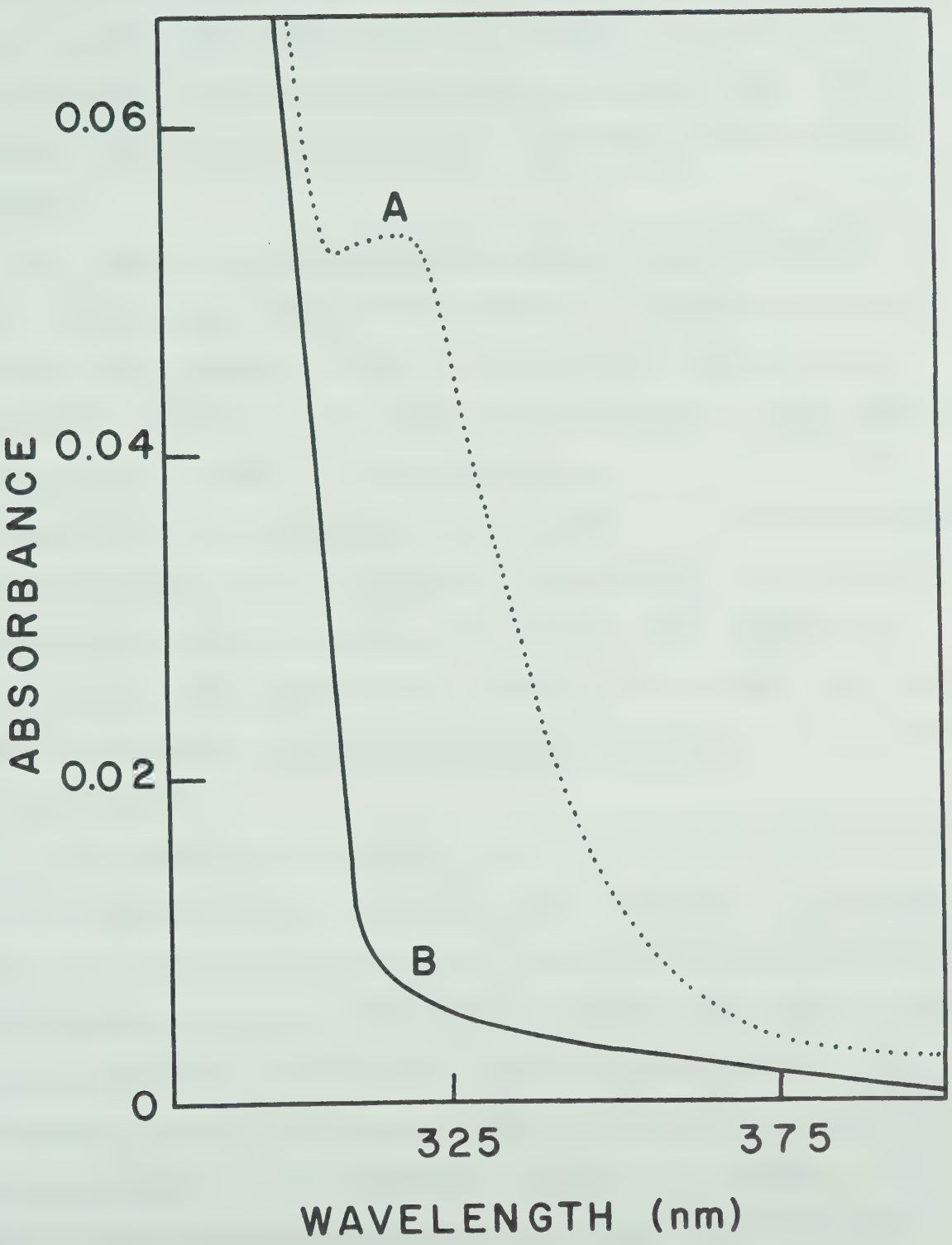
Absorption spectra of apophosphorylase b and DHT-treated apophosphorylase b. Samples were prepared as described in Table XVII.

A: DHT apophosphorylase b, 0.65 mg/ml.

B: Apophosphorylase b, 0.57 mg/ml.

Both samples in 50 mM GP, 50 mM ME, 1.5 mM EDTA, pH 6.8.

Each blank contained only this buffer.


0.06
0.04
0.02
0
ABSORBANCE
A
B
325
375
WAVELENGTH (nm)

On the other hand, retention of the 321 nm peak and about half of the isotope suggests that at least part of the DHT modifies an amino acid residue. In order to examine the relationship of these possibilities an attempt was made to remove the isotope from a protein where the PLP was firmly bound.

4. Removal of PLP from DHT-treated Phosphorylase b

IAM phosphorylase b was prepared. A portion of this protein was reduced. Both the IAM phosphorylase b and reduced IAM phosphorylase b were passed through a G-25 Sephadex column into 50 mM P_i, 1.5 mM EDTA, pH 6.1. Both protein samples, at a concentration of 8.3 mg/ml, were treated with 0.336 mM DHT at 0°C. A sample at one minute was taken for an activity assay, and then the reaction was terminated. DHT-treated IAM phosphorylase b had a 20% residual activity, while DHT-treated reduced IAM phosphorylase b had a 12% residual activity.

The DHT-treated proteins and controls were passed through a G-25 Sephadex column into 20 mM GP, 5 mM ME, 1.5 mM EDTA, pH 6.8. Appropriate studies on these samples indicated that IAM phosphorylase b incorporated 0.855 DHT per enzyme monomer while remaining 93% dimer, S = 8.6 and 7% monomer, S = 6.1. However, reduced IAM phosphorylase b incorporated 2.05 DHT per enzyme monomer, with 48% dimer, S = 8.6, 31% monomer, S = 6.1, and a fast-sedimenting peak, S = 15.9 with an area of 21%.

Absorption spectra were obtained of the DHT-treated samples. Both samples gave a peak at 320 nm with a shoulder

at 375 to 400 nm. Excitation at 320 nm of DHT IAM phosphorylase b resulted in a 535 nm fluorescence peak, while the same excitation of the DHT-treated reduced protein resulted in a 400 nm fluorescence peak indicative of PMP. Therefore, two types of DHT treated proteins had been prepared. In one case (reduced enzyme) the cofactor was firmly bound to the enzyme, in the other (native enzyme) the cofactor was more labile. This presented an opportunity to remove the cofactor from one protein and not the other. If the loss of the isotope was dependent upon cofactor removal, then SDS treatment of the reduced enzyme, where the PMP was firmly bound, should not result in loss of the isotope; however, SDS treatment of native protein, where the PLP is labile, should result in loss of the isotope concomitant with the removal of the cofactor.

Both DHT-treated IAM phosphorylase b and its control were treated with 2% SDS. Absorption spectra of the samples showed the release of unmodified PLP. These results are shown in Figure 17. Emission spectra of these same proteins also indicated that SDS treatment had released an unmodified PLP. These samples were dialyzed for 12 hours against small volumes of 20 mM GP, 5 mM ME, 1.5 mM EDTA, pH 6.8. Emission and absorption spectra revealed that the control and DHT-treated protein both lost the cofactor. However, the DHT-treated protein retained its 320 nm peak and 0.66 moles of DHT per enzyme monomer. Spectral examination of the dialysate revealed that both proteins had released unmodified cofactors.

Figure 17

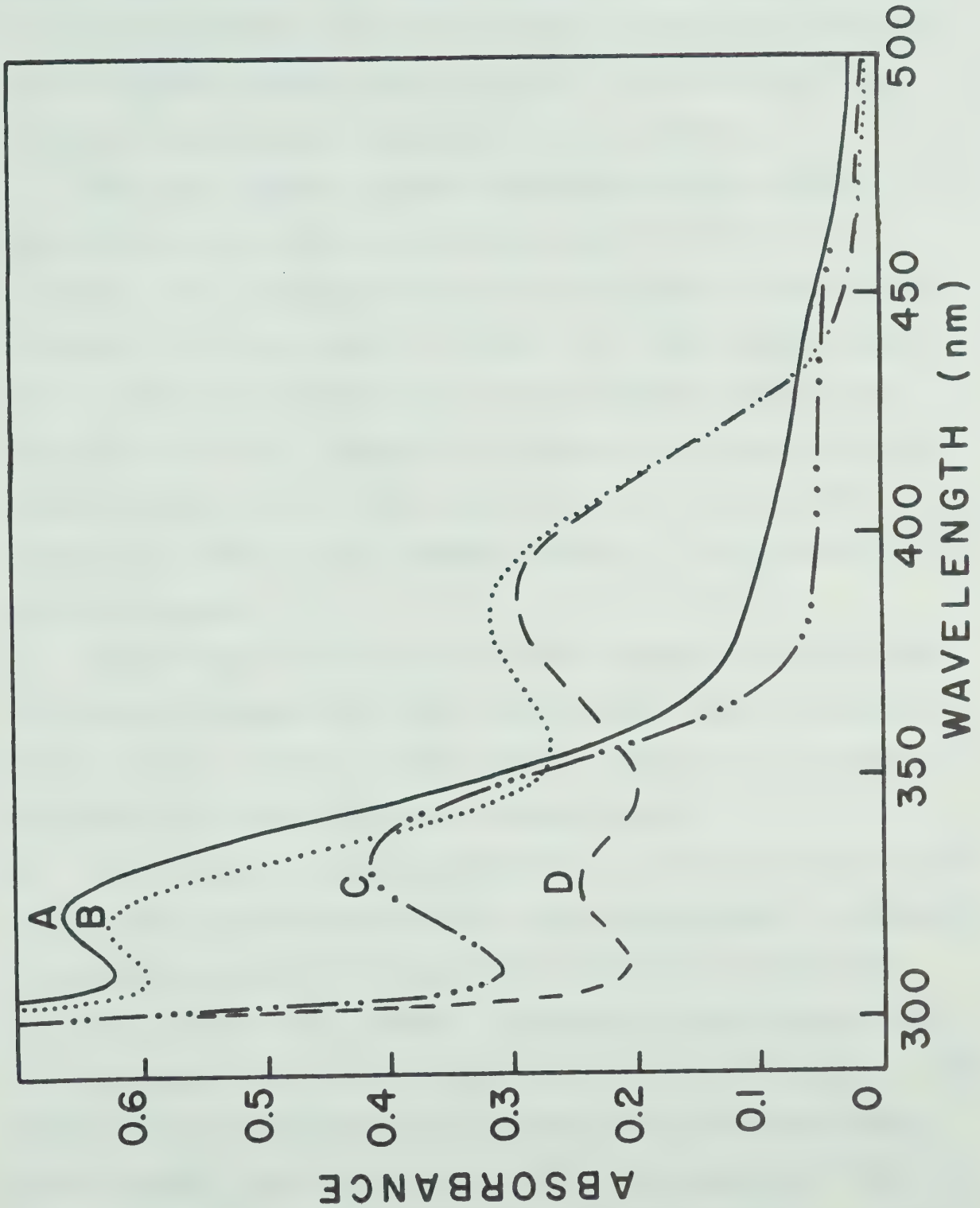
Absorption spectra of IAM phosphorylase b and DHT IAM phosphorylase b before and after treatment with 2% SDS. All samples were in 20 mM GP, 5 mM ME, 1.5 mM EDTA. Blanks for samples A and C contained only the buffer, while blanks for samples B and D contained buffer plus 2% SDS.

A: DHT IAM phosphorylase b, 5.3 mg/ml, pH 6.8.

B: DHT IAM phosphorylase b plus 2% SDS, 5.3 mg/ml, pH 7.0.

C: IAM phosphorylase b, 6.5 mg/ml, pH 6.8.

D: IAM phosphorylase b plus 2% SDS, 5.2 mg/ml, pH 7.0.


ABSORBANCE
0.6
0.5
0.4
0.3
0.2
0.1
0
300
350
400
450
500
WAVELENGTH (nm)
A
B
C
D

After these studies, the SDS treated DHT IAM phosphorylase b was exhaustively dialyzed (3 buffer changes over 59 hours, with a buffer:protein ratio of 50:1). This dialysis resulted in the protein losing 41% of its isotope.

Both DHT-treated reduced IAM phosphorylase b and its control were treated in the same manner as the native enzymes. SDS treatment produces an increase in absorbance at about 325 nm as shown in Figure 18. This results from the shifting of the PMP from its hydrophobic pocket upon denaturation (37). Exhaustive dialysis (two buffer changes over 44 hours with a buffer:protein ratio of 50:1) did not remove any PMP from the enzyme, although it did remove 42% of the isotope.

Molar absorption values for DHT-treated proteins before and after treatment with SDS and dialysis were calculated. These results, shown in Table XVIII, indicate that similar residues are modified in both enzyme forms.

These results may be summarized in the following manner. First, SDS treatment of the DHT IAM phosphorylase b followed by dialysis results in the protein losing all of its PLP, but only part of its isotope. Second, the PLP removed by SDS treatment and dialysis was not modified. Therefore, DHT does not react with PLP, but instead must modify an amino acid residue. Also the isotope which was removed at the same time as the PLP must be due to the breakage of a labile bond of the DHT modified amino acid. Third, similar treatment of the DHT reduced IAM phosphorylase b does not cause any loss

Figure 18

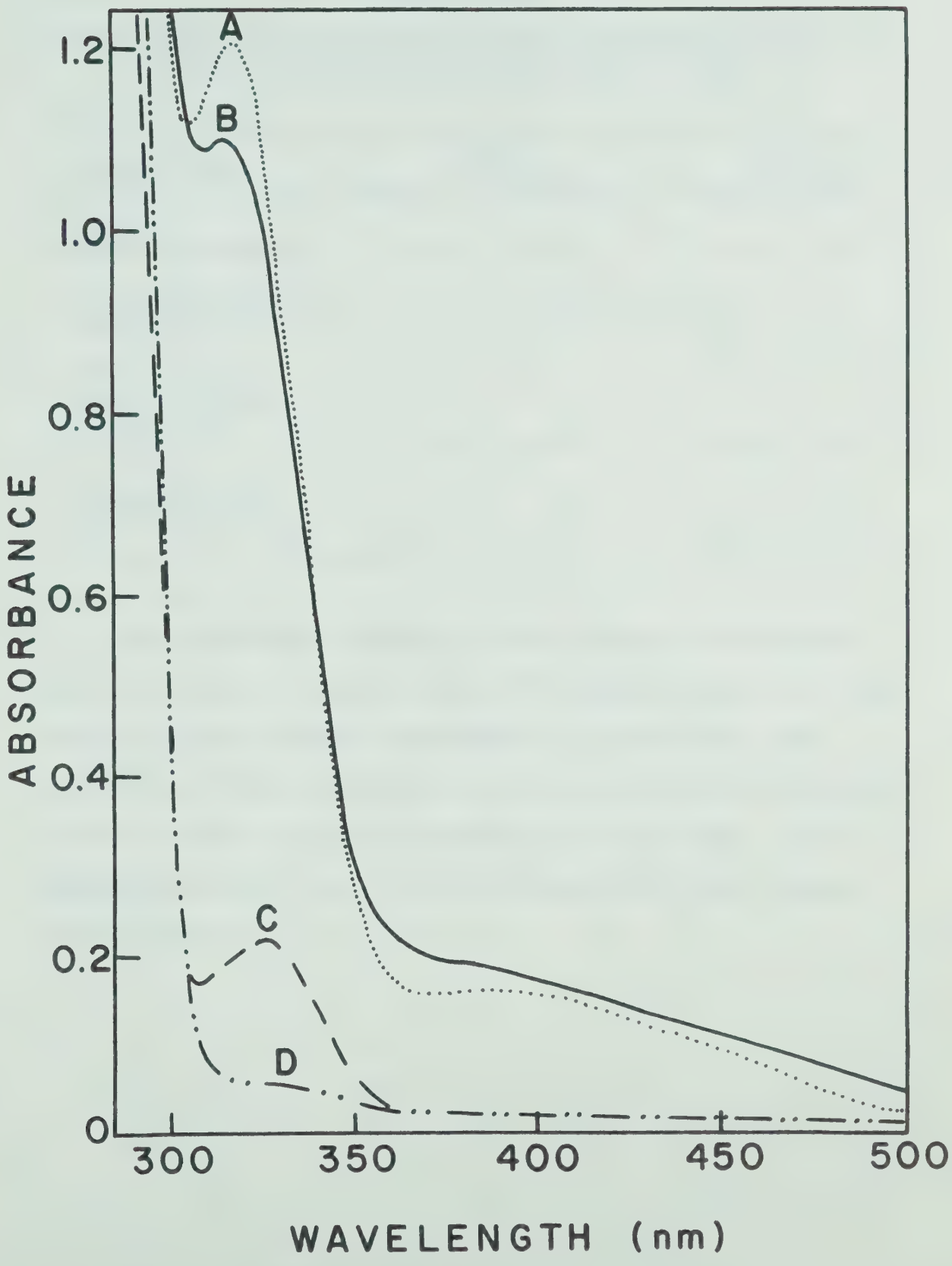
Absorption spectra of reduced IAM phosphorylase b, and DHT reduced IAM phosphorylase b, before and after treatment with 2% SDS. All samples were in 20 mM GP, 5 mM ME, 1.5 mM EDTA. Blanks for samples B and D contained only this buffer, while blanks for samples A and C contained buffer plus 2% SDS.

A: DHT reduced IAM phosphorylase b plus 2% SDS, 4.9 mg/ml, pH 7.0.

B: DHT reduced IAM phosphorylase b, 4.9 mg/ml, pH 6.8.

C: Reduced IAM phosphorylase b plus 2% SDS, 2.2 mg/ml, pH 7.0.

D: Reduced IAM phosphorylase b, 2.3 mg/ml, pH 6.8.


A
B
C
D
1.2
1.0
0.8
0.6
0.4
0.2
0
ABSORBANCE
300
350
400
450
500
WAVELENGTH (nm)

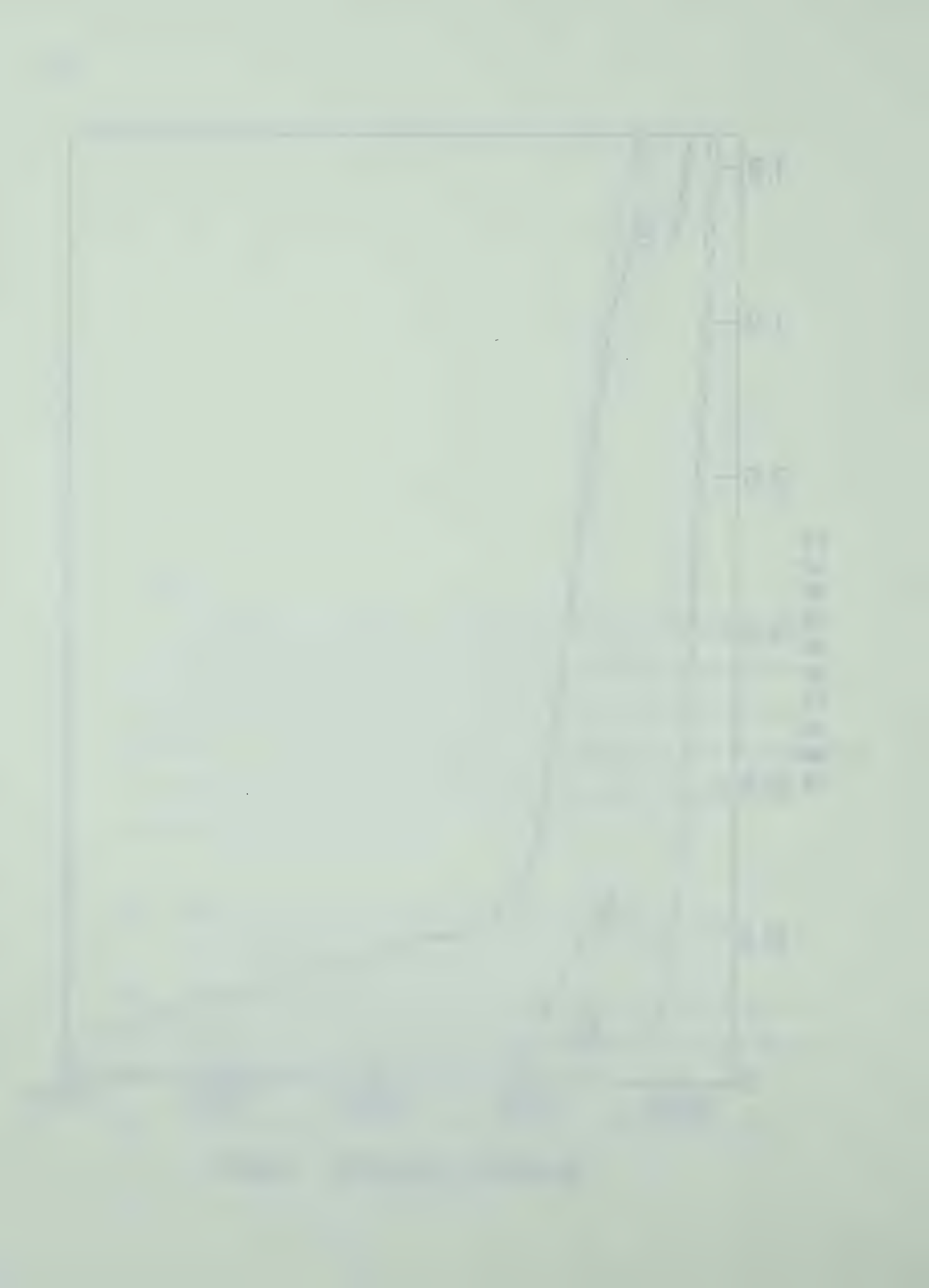


TABLE XVIII

Molar Absorptivity Values for DHT IAM Phosphorylase b Before and After Treatment with 2% SDS

Enzyme Form	ε at 320 nm	ε at 387 nm
DHT IAM phosphorylase b	8.7×10^3	1.15×10^3
DHT reduced IAM phosphorylase b	1.05×10^4	1.3×10^3
DHT IAM phosphorylase b SDS treated and dialyzed	7.6×10^3	7.05×10^2
DHT reduced IAM phosphorylase b	1.14×10^4	1.41×10^3

The absorption values at the indicated wavelength were measured for each of the DHT-treated proteins. The contribution from the corresponding control was subtracted and the resulting value was corrected to represent 1 mole of DHT per mole of monomer of the enzyme. This absorption value was used to calculate the molar absorptivity value.

of PMP, but does release some of the isotope. Therefore, removal of the isotope is independent of the loss of the cofactor. Fourth, both DHT-treated native and reduced enzyme show similar molar absorptivity values and a similar percentage of labile isotope. Therefore, it appears that the same residues are modified in both proteins.

5. Reconstitution of DHT IAM phosphorylase b

Samples of DHT IAM phosphorylase b and IAM phosphorylase b were resolved as described in Materials and Methods. The resulting precipitated apoproteins were redissolved in 50 mM GP, 50 mM ME, pH 7.0, then passed through a G-25 Sephadex column into the same buffer. An absorption spectrum of apo DHT IAM phosphorylase b, shown in Figure 19, revealed a 320 nm peak and 387 nm shoulder, with molar absorptivity values of 1.43×10^4 and 2.69×10^3 respectively. Excitation of both apoprotein samples at 320 nm resulted in small 535 nm peaks. While the apo DHT protein had no enzymic activity, the apo control sample had 13% residual activity, thus indicating that 87% of the enzyme was resolved.

Both apo DHT IAM phosphorylase b and apo IAM phosphorylase b were reconstituted with PLP and the enzymic activities examined. While the reconstituted control regained 92% of its former activity, the apo DHT IAM protein only regained 25%. Radioactive counting of the reconstituted DHT-treated protein revealed that it lost 53% of its isotope, retaining 0.40 moles of DHT per mole of enzyme monomer.

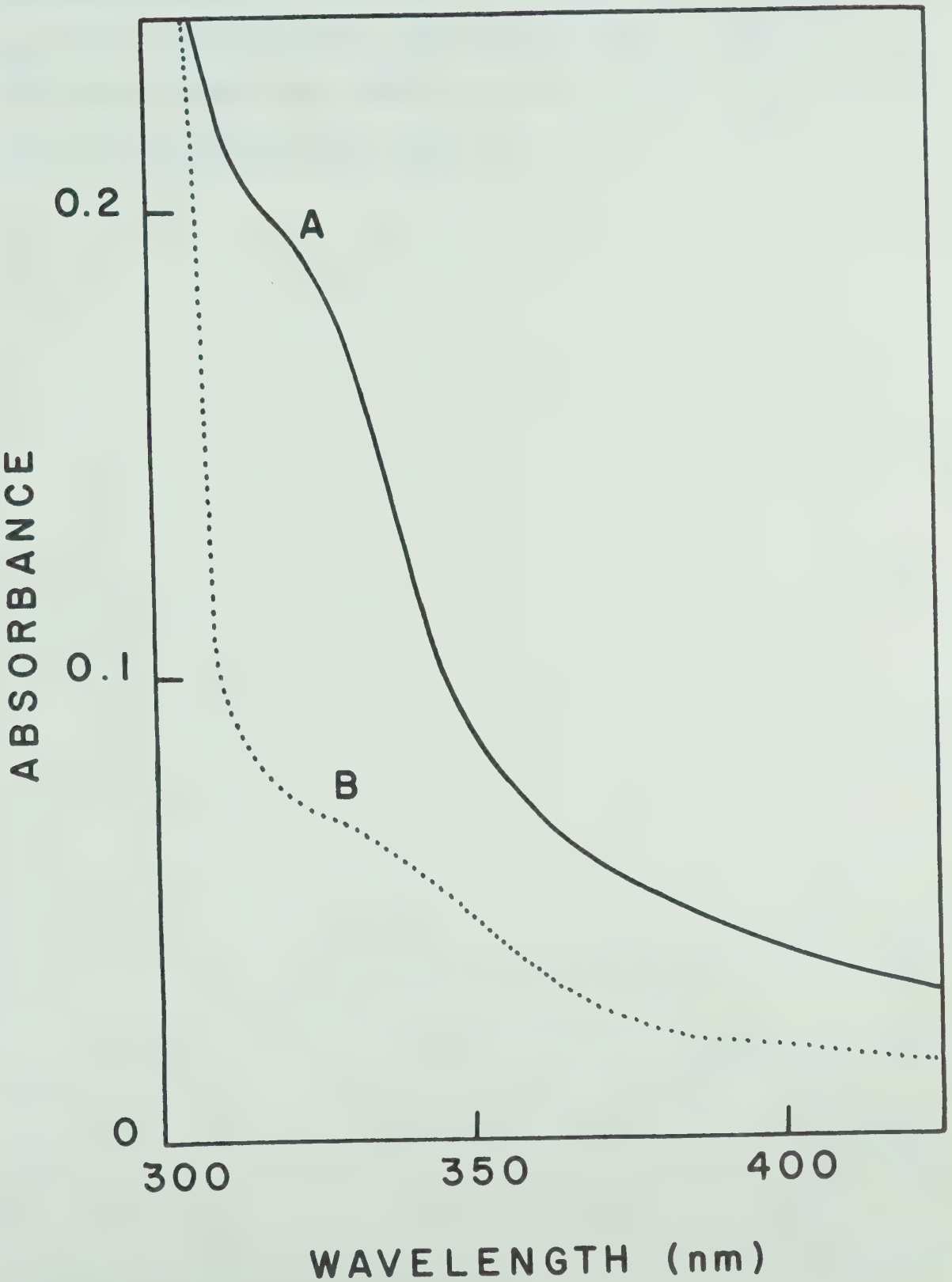
Since reconstitution of the protein does not return

Figure 19

Absorbance spectra of apo IAM phosphorylase b and apo DHT IAM phosphorylase b. Both samples were in 50 mM GP, 50 mM ME, pH 7.0. Blanks contained only this buffer.

A: Apo DHT IAM phosphorylase b, 2.7 mg/ml.

B: Apo IAM phosphorylase b, 2.2 mg/ml.


0.2
0.1
0
ABSORBANCE
A
B
300
350
400
WAVELENGTH (nm)

enzymic activity, a modified PLP in the DHT IAM phosphorylase b is not responsible for the activity loss. Retention of the 320 nm peak after removal of all the PLP indicates that amino acid residues are modified.

D. Peptide Analysis

Pepsin and trypsin digests were carried out on DHT treated phosphorylse b samples. The first samples examined were from the radioactive DHT inactivation experiment reported in Part III, Section B (see Table XI). A DHT IAM phosphorylase b sample was selected which showed 7% residual activity with 2.58 moles of DHT incorporated per mole of monomer of the enzyme. This protein was lyophilized and stored below 0°C for two months. Then it was prepared for both a trypsin and pepsin digest. Treatment of the protein with 5% formic acid with subsequent dialysis against 5% formic acid resulted in 60-70% of the radioactive counts being removed from the enzyme. Treatment of the DHT protein with trypsin and removal of the precipitate also resulted in a 60-70% loss of the isotope. The peptide mixtures were separated on paper by high voltage electrophoresis at pH 6.5. The positions of the radioactive peptides were determined by scanning the electropherogram. The trypsin digest results gave at least two peptides in the neutral region. The electropherogram of the pepsin digest indicated one neutral and one acidic peptide.

A pepsin digest was carried out on the DHT-treated IAM phosphorylase b (0.92 moles DHT per monomer of protein) prepared for the spectral experiment (see Part IV, Section B). Treatment of this protein with 5% formate and dialysis against the same resulted in 49% of the radioactive counts being removed from the enzyme. After separation on paper by

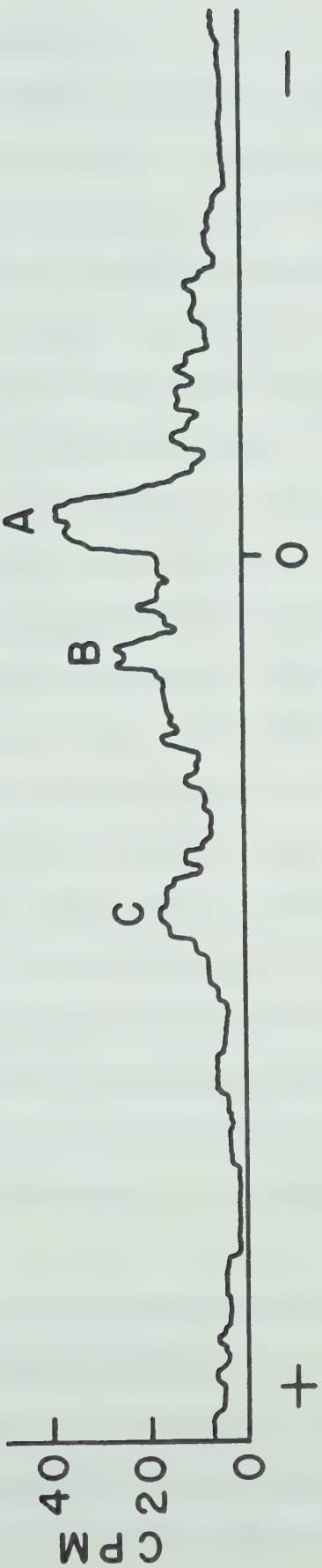
high voltage electrophoresis at pH 6.5, the electropherogram was scanned to determine the positions of the radioactive peptides. The resolution of these results was better than in the previous example, indicating at least three different peptides.

A pepsin digest was done on both DHT-treated IAM phosphorylase b and DHT treated reduced IAM phosphorylase b from the PLP experiment (see Part IV, Section C). Treatment with 5% formate followed by dialysis against the same resulted in the former sample losing 53% and the latter sample losing 51% of its radioactive isotope. Hence a pepsin digest was carried out on DHT IAM phosphorylase b containing 0.40 moles of DHT per mole of enzyme monomer, and on a DHT reduced IAM phosphorylase b containing 1.00 moles of DHT per mole of enzyme monomer. The resulting peptides were separated by high voltage electrophoresis at pH 6.5. The electropherograms were scanned to determine the number and position of the radioactive peptides. The DHT IAM phosphorylase b samples showed the characteristic three peptides. The results are shown in Figure 20. The DHT reduced IAM phosphorylase b sample showed six radioactive peptides in the neutral and acidic regions.

These results indicate the difficulty of identifying the reactive residue in the protein by peptide isolation, since identification of only 0.5 modified residues per enzyme monomer would require isolation of three peptides.

Figure 20

Position of peptides containing radioactive DHT. IAM phosphorylase b containing 0.40 moles of DHT per mole of monomer of the enzyme was made 5% with respect to formic acid and then exhaustively dialyzed against 5% formic acid. Pepsin was added to the protein (1 mg per 10 mg phosphorylase) and the sample incubated for 24 hours at 37°C. 0.68 mg of the protein was applied to a one cm diameter spot on #1 paper, then electrophoresis carried out at pH 6.5 for 45 minutes at 3000 volts. A strip was cut out which included one cm on either side of the original spots to include the entire amount of protein. Both strips were put through the Actigraph III scanner at 15 cm per hour.


CPM
40
20
0
+
0
−
A
B
C

DISCUSSION

DHT has been used to examine tyrosine, histidine and lysine residues in numerous proteins. These proteins were treated, normally at room temperature, with a large excess of the reagent at alkaline pH in order to achieve the desired inactivation. Alkaline pH values were employed for two reasons. First, it was assumed that the reactivity of the residues under investigation would be larger at higher pH values. Second, the molar absorptivity values of DHT derivatives of tyrosine and histidine were obtained at pH 8.8 (6, 7). The reasons for using room temperature as opposed to 0°C were never stated. Why large excesses of DHT were necessary to inactivate these proteins was also never explained. However, it may be inferred from the results presented in Figures 1 and 3 that the rapid hydrolysis of DHT was part of the reason. Another reason may have been the lack of specificity of DHT. As demonstrated in Table II, DHT may react with eight amino acids. Therefore, those studies which identify modified histidine, tyrosine and lysine residues by spectra and amino acid analysis, but fail to determine the total number of modified residues through the use of radioactive DHT, are of dubious value.

The conditions employed for DHT treatment of phosphorylase were found important for determining the per cent inactivation and specificity of the reaction. Table III suggests that inactivation of phosphorylase b at room temperature is independent of pH. However, when the temperature is lowered

to 0°C, the pH at which the reaction is carried out definitely is important, as indicated by the titration experiments. A higher concentration of DHT must be used at pH 8.8 to obtain the same inactivation observed at pH 6.1 with a lower concentration. The reason for this is that the specificity of the DHT reaction with phosphorylase is controlled by the pH at which the reaction is conducted. Figure 6 demonstrates that DHT treatment of phosphorylase a and b at 0°C and pH 6.1 modifies either the same residues or residues with similar spectral properties. Radioactive DHT treatment of phosphorylase b shows that, when the time of reaction is limited, only residues essential for catalytic activity are modified. However, DHT treatment of phosphorylase a or b at 0°C, pH 8.8, and high concentration of reactants, results in modification of both essential and non-essential residues. Molar absorptivity values presented in Table XII show that the essential residues of phosphorylase b modified at pH 8.8 are different from those modified at pH 6.1 in both native and reduced phosphorylase b. Therefore, at least with phosphorylase b, at 0°C, the pH at which the reaction is conducted does determine its specificity.

The effect that temperature has on the reaction between DHT and phosphorylase was not fully examined. With phosphorylase b, treatment at both room temperature and 0°C produced similar results, although the former required more DHT for the same per cent inactivation observed with the latter. This was due to the rapid hydrolysis of DHT at room temperature. Under both conditions a DHT-treated protein may be produced which

is totally inactive, remains predominantly dimer, and has a 320 nm peak with a 375 - 400 nm shoulder. These results suggest that at pH 6.1 the specificity of the reagent is the same at both temperatures. Whether or not the specificity of the reagent is the same at pH 8.8 at both temperatures was not examined.

Conditions were sought whereby the per cent inactivation could be maximized at the same time that the number of modified residues were minimized. Comparison of the titration experiments and the spectral results summarized in Table XII indicate that DHT treatment of phosphorylase a or b at pH 6.1, 0°C, would produce the desired results. Figure 6 indicates that phosphorylase b was inactivated with fewer modified residues.

Therefore, radioactive DHT was used to treat phosphorylase b at 0°C, pH 6.1, in order to determine the number of residues modified and their relation to catalytic activity. Examination of the results presented in Table XI and Figure 12 revealed that (1) modifying one residue within the first minute would result in a total loss in activity and (2) DHT incorporated after the first minute did not result in a corresponding loss in activity. This latter result explains why the activity of the protein drops during the first minute, but then remains essentially constant during the remaining 30 minutes (see Figure 7), even though it is known that sufficient DHT is present to continue the inactivation. When the concentration of the reactants is lowered sufficiently a small reduction

in the rate of inactivation is observed, as indicated in Figure 9. The radioactive titration shown in Figure 13 reveals that under these conditions an increase in incorporation after the first minute does produce a decrease in the per cent residual activity.

It appears that residues not essential to catalytic activity react with DHT after the residue related to catalytic activity is modified. In other words, upon addition of DHT to the protein a residue X is modified within the first minute. Complete modification of this residue (one per monomer) would result in total inactivation. However, modification of X produces a conformational change in the enzyme resulting in a new state, where residues not related to catalytic activity are more reactive with DHT in comparison to the remaining unreacted X residues. Therefore, before the complete population of X residues may be titrated, a number of new residues not related to catalytic activity are modified by DHT. Hence, DHT incorporation after the first minute does not result in a corresponding loss in activity. However, when the concentration of the reactants is lowered, then the reaction rate of X is slowed sufficiently so that these residues continue to be modified after one minute.

Therefore, in order to ensure that only residues essential to catalytic activity are modified, phosphorylase b must be treated while controlling the concentration of reagents, pH, temperature and the length of time that the reaction

proceeds. When this protein at 5 - 10 mg/ml was treated with 0.3 to 0.5 mM DHT, at pH 6.1, 0°C, for one minute, between 0.8 to 1.2 residues per enzyme monomer were modified, resulting in 70 - 100% loss in activity.

It appears that this modified residue represents only one amino acid, and not portions of two or more. This is supported by the results shown in Table XVIII. Removal of the labile isotope from the DHT-treated phosphorylase b does not change the molar absorptivity values. This shows that only one amino acid is modified.

Since only one amino acid residue is modified, it is surprising that three peptides are found containing the modified residue. It would appear possible that all three of these are variations of the same peptide, produced by the lack of specificity of pepsin. It is also surprising that only 50% of the isotope is removed after treatment with SDS or strong acid. However, possibly all of the isotope is labile. It was shown in Part IV, Section C, that SDS treatment of DHT phosphorylase b followed by dialysis for a specified length of time resulted in about a 50% loss of the isotope. However, continued dialysis led to removal of more isotope, but the amount could not be determined since the protein precipitated. Therefore, exhaustive dialysis of either SDS or strong acid treated DHT protein may remove all the isotope.

The quenching experiments in Part III, Section A, also suggest that under harsh conditions all the isotope may be

released from the protein. The evidence presented establishes that there is no quenching in spite of the fact that a precipitate is formed upon addition of the protein to the scintillation fluor. If the isotope remains in the precipitated protein then some quenching would have to be observed. The lack of any quenching suggests that the strong organic solvents denature and precipitate the protein, releasing the isotope into the supernatant.

Therefore, it appears that DHT modifies one residue per phosphorylase b monomer resulting in total inactivation. An attempt was made to elucidate the nature of this residue.

Comparison of the model compound studies presented in Part I, Section C, and the spectral experiment shown in Part IV, Section B, demonstrated that the modified residue is not a tyrosine, histidine, lysine or tryptophan.

The DHT modified residue in phosphorylase b produced a 320 nm peak and 387 nm shoulder, with molar absorptivity values of about 1.0×10^4 and 2.0×10^3 respectively. These molar absorptivity values agree with that of monoazotetrazole-N-acetylcysteine. However, DHT-treated phosphorylase b remains predominantly a dimer and contains reactive B_1, B_2, N and A sulfhydryl residues. Therefore, these residues are not modified by DHT. The remaining sulfhydryl residues are buried in the protein and are not reactive with normal sulfhydryl reagents (38).

Absorption and emission spectra, along with the simultaneous loss of isotope and PLP, suggested that DHT directly

modified the cofactor. However, a number of experiments demonstrated that PLP was not the titrated residue. Incorporation values for DHT-treated phosphorylase b were the same as for DHT-treated apophosphorylase b. Reconstituted DHT-treated apophosphorylase b only regained 25% of the control activity. Finally in Part IV, Section C, it was shown that the PLP removed from the DHT-treated enzyme was not modified, and that the 50% loss of isotope concomitant with total loss of cofactor was due to a labile bond in the monoazotetrazole amino acid.

Therefore, it appears that DHT may react with one of the three remaining residues--arginine, serine or threonine. Since their N-acetyl derivatives react slowly with DHT, no molar absorptivity values could be obtained. Thus one can only speculate as to which may be the reactive residue. Arginine would seem less likely than the other since Fukui et al. have shown that phosphorylase b does not contain any highly reactive arginines which are important for catalytic activity (19). Positive identification of the residue must await isolation of the modified peptide.

Whether or not the modified residue is at the active site cannot be determined from the data presented. As shown in Table XI the substrates afford little or no protection. However, in view of the large Km for the substrates and high reactivity of the residue, little can be inferred from such protection studies.

In summary, this reagent will inactivate phosphorylases.

Under specific conditions, DHT treatment of phosphorylase b modifies one unidentified amino acid residue, producing 100% loss in activity.

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